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PRINCIPAL INVESTIGATOR: Sally Kornbluth, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
                           Durham, North Carolina 27710

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Sally Kornbluth, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: kornb001@mc.duke.edu

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13. ABSTRACT (Maximum 200 Words)
Apoptosis is a program of cellular suicide in which individual cells are removed from the midst of a living tissue without damaging overall tissue architecture. In recent years it has been recognized that tumor formation results not only from an increase in cellular proliferation, but also from a failure of cells to die by apoptosis. Moreover, many chemotherapeutic agents are now believed to act through induction of apoptosis. Hence, an understanding of basic mechanisms of apoptosis should aid in the treatment of a variety of tumors. The work funded by this proposal utilized a novel cell-free assay derived from Xenopus eggs to study the biochemical mechanisms underlying apoptotic progression. Using this system we have uncovered a number of novel apoptotic regulators, as well as demonstrating that apoptosis can be inhibited at several points after initiation of a death signal. In particular, we have found that several different kinase signaling pathways can prevent induction of cell death very late in the process, after a number of important apoptotic regulators have already transited from the mitochondria to the cytoplasm. Specifically, MAP kinase pathways and the Bcr/Abl tyrosine kinase can both act to prevent processing and activation of death proteases (the caspases) even after a caspase co-activator, cytochrome c, has exited the mitochondria.

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Figures + 5 manuscripts appended

Introduction

Apoptosis is a program of cellular suicide which eliminates harmful or superfluous cells without damaging neighboring tissue. Research into the biochemistry of apoptosis promises to broadly impact the treatment of cancer. Specifically, the inability of breast cancer cells to undergo appropriate cell death may contribute to both tumor formation and resistance to chemotherapetic and radiologic treatments.

The key cell executioners in the apoptotic program are a family of proteases known as caspases[1]. To some extent apoptotic signaling can be understood as a series of events leading to caspase activation. Caspases are synthesized as pro-enzymes which must be cleaved prior to proteolytic activation. Numerous lines of evidence support the notion that the pro-domains of caspases, removed upon proteolytic activation, serve as binding sites for critical regulatory proteins which determine whether or not activation of the pro-caspases will occur [2-4]. In our research, we aim to utilize an in vitro apoptotic system based on Xenopus egg extracts to explore the role of caspase pro-domain-interacting proteins in regulation of the cell death program. Our goal has been to identify and purify these binding proteins in the hopes of identifying novel apoptotic regulators. Ultimately, we will assess the roles of these regulators in both our in vitro system and in breast cancer cells.

Body of Report:

The major objective of this work was to isolate regulators of caspase activation using our in vitro Xenopus egg extract system. As this is the final report, I will summarize below the key accomplishments steming from the three years of funding. After extensive experimentation, we found that we could not proceed exactly as planned in our original proposal. Specifically, we had proposed to isolate proteins interacting with the pro-domains of caspases using recombinant GST-caspase fusion proteins as baits to retrieve apoptotic regulators from Xenopus egg extracts. This objective (**Technical objective I**) proved difficult as the recombinant pro-domains did not "behave" well as recombinant baits, tending to precipitate out of solution. Even when soluble, they were generally inefficient at retrieval of bound proteins. Hence, we focused most of our efforts in this funding period on **technical objectives II and III**, which involved characterizing apoptotic stimuli in the in vitro system and isolation of novel upstream regulators of pro-caspases. These approaches have proven quite successful, allowing us to identify and characterize a number of novel apoptotic regulatory pathways.

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MAP kinase pathways and prevention of apoptosis

In characterizing pro-caspase activation in our extracts, we noted that M phase-arrested extracts (CSF Extracts) were considerably more refractory to apoptosis than were the equivalent extracts arrested in interphase (S extracts). Surprisingly, however, this difference in apoptotic potential was not reflected in the level of mitochondrial cytochrome c release, normally a correlate of apoptotic progression in the egg extract [5-6]. Specifically, cytochrome c was released from mitochondria in M phase extracts, but caspase activation did not occur. This suggested that M phase extracts might have a mechanism to prevent caspase activation even after cytochrome c release from mitochondria. Moreover, as the Xenopus extracts do not transcribe mRNAs, this post-cytochrome c protection from apoptosis had to be entirely post-translational. As M extracts have high levels of both Cdc2/Cyclin B and MAPK activity, we looked at the effects of Cdc2 inhibitors and MAPK pathway inhibitors on the ability of extracts to resist cytochrome c-induced caspase activation. Intriguingly, we found that inactivation of Cdc2 had no effect on apoptotic progression, while removal of MEK/inactivation of the Erk pathway entirely abrogated the post-cytochrome c protection from apoptosis. In keeping with this, ectopic addition of thiophosphorylated Erk to interphase cytosol conferred protection against added cytochrome c, as did addition of either Mek or Mos, an upstream activator of the MAPK pathway. This work is described in detail in the appended manuscript by Tashker et al. which is in press in *Molecular Biology of the Cell*.

In pursuing the mechanism of MAPK-mediate post-cytochrome c protection from apoptosis, we have conformed closely to the methodolgy described in **technical objectives II and III** of the original proposal. However, instead of using caspase pro-domains as the "handle" to pull out caspase regulators, we have used cytochrome c immobilized on a sepharose resin. Reasoning that this resin should pull out the caspase regulator Apaf-1, along with associated caspase 9 and any additional regulators, we incubated the resin with either interphase or M phase extract, pelleted and washed the beads and looked for processing of exogenously added pro-caspase 9. As shown in Fig. 1., cytochrome c-bound "apoptosomes" from M phase extracts(UCSF) were considerably less efficient than apoptosomes retrieved from S phase extracts (US) at processing added pro-caspase 9. Moreover, activation of MAP kinase pathways in interphase extracts by addition of the MEK activator Mos also led to apoptosomal inhibition. An inactive variant of Mos had no such effect. More recently, we have identified a coomassie blue-stainable band that associates with the apoptosome from interphase, but not M extracts or interphase extracts treated with Mos. We are currently microsequencing this protein. Characterization will proceed according to methods very similar to that described for pro-domain-binding partners in the original proposal.

Bcr/abl and the inhibition of apoptosis

As described last year, and originally described in **Technical objective I, task 3,** we found that recombinant Bcr/Abl (an oncogenic tyrosine kinase) could prevent apoptosis upon addition to Xenopus egg extracts. Intriguingly, we have now found that Bcr/Abl can prevent apoptosis both by preventing mitochondrial cytochrome c release and by preventing activation of pro-caspase 9 by any cytochrome c that does move to the cytoplasm. Indeed, addition of cytochrome c to pure cytosol triggers robust activationof pro-caspase 9, which can be effectively inhibited by addition of Bcr/Abl (Fig. 2). In accordance with this, Bcr/Abl also prevents activation of the downstream effector caspase, caspase 3 (Fig. 3) and this caspase 3 inhibition requires that Bcr/Abl be catalytically active. Intriguingly, post-cytochrome c protection by Bcr/Abl appears to operate by a mechanism entirely distinct from that of MAP kinase pathway activation as it is not prevented by MEK inhibitors; moreover, we have shown that Bcr/Abl added to the extract does not activate MAP kinase pathways in the way that Mos did. (Fig. 4).

Crk-induced apoptosis

Before starting this work, we had shown that the adaptor protein Crk, consisting of an SH2 domain and two SH3 domains, was required for apoptotic signaling in the egg extract [6]. Recently, we have extended this work to demonstrate that Crk is able to regulate apoptosis in mammalian cells. Moreover, we have identified a novel nuclear export sequence in Crk within the C-terminal SH3 domain of the protein. Mutations within the export sequence enhance the apoptotic potential of the Crk protein, suggesting that nuclear Crk is pro-apoptotic. Consistent with this, we have found that Crk can bind to the nuclear tyrosine kinase Wee1 in an interaction required for apoptotic signal transmission. This work is described in the appended manuscript by Smith et al., currently in press at *Molecular and Cellular Biology*.

Reaper-induced apoptosis

As originally described in **Technical objective I, task 3,** we wished to examine potential caspase activators in the presence and absence of the apoptotic regulator Reaper. Extensive work in our laboratory has identified a novel regulator of Hsc 70-mediated protein folding, Scythe, as a mediator of Reaper-induced cytochrome c release from mitochondria. The Scythe work, which grew out of Reaper characterization in Technical objective I grew into a distinct project (no overlap), tangentially related to our original application. Hence, the groundwork laid in the research funded by the IDEA award led to a novel project funded by the NIH. This work has resulted in the publication of 3 papers (appended) in *EMBO J.* Details of this work can be found in the appended manuscripts.

Key research accomplishments:

- Identification of a novel apoptotic regulatory pathway acting downstream of MAP kinase activation to prevent apoptosis after mitochondrial release of cytochrome c.

- Identification of a post-cytochrome c protection from apoptosis operating downstream of the oncogenic tyrosine kinase Bcr/Abl.

- Establishment of a system to study Reaper-induced apoptosis in Xenopus egg extracts, leading to a novel line of research identifying Scythe as a downstream effector of Reaper.

- Further characterization of Crk-regulated apoptosis, leading to the identification of a novel means of regulating Crk and apoptotic progression through nuclear export.

Reportable outcomes:

Two manuscripts are currently in press resulting from this (Tashker et al. and Smith et. al, appended). The initial work on Reaper funded by this grant gave rise to a novel line of research resulting in funding of an NIH RO1. This work has given rise to a series of EMBO J papers which are appended [7-9]. Three additional papers (one on post-cytochrome c protection by Bcr/Abl and two on Reaper) are currently in preparation for publication.

Conclusions:

The long term goal of the original proposal was to isolate novel regulators of apoptosis through the characterization and isolation of key apoptotic modulators from our in vitro apoptotic reconstitution system. Through this work, we have identified a new mechanism of apoptotic regulation, prevention of cytochrome c-mediated caspase activation. We have shown that Bcr/Abl and MAP kinase pathways can both utilize this mechanism to prevent apoptosis. Moverover, using our cytochrome c-bound apoptosomes, we now have the means to identify the proteins differentially associated with apoptosomes in the presence and absence of MAPK or Bcr/Abl.
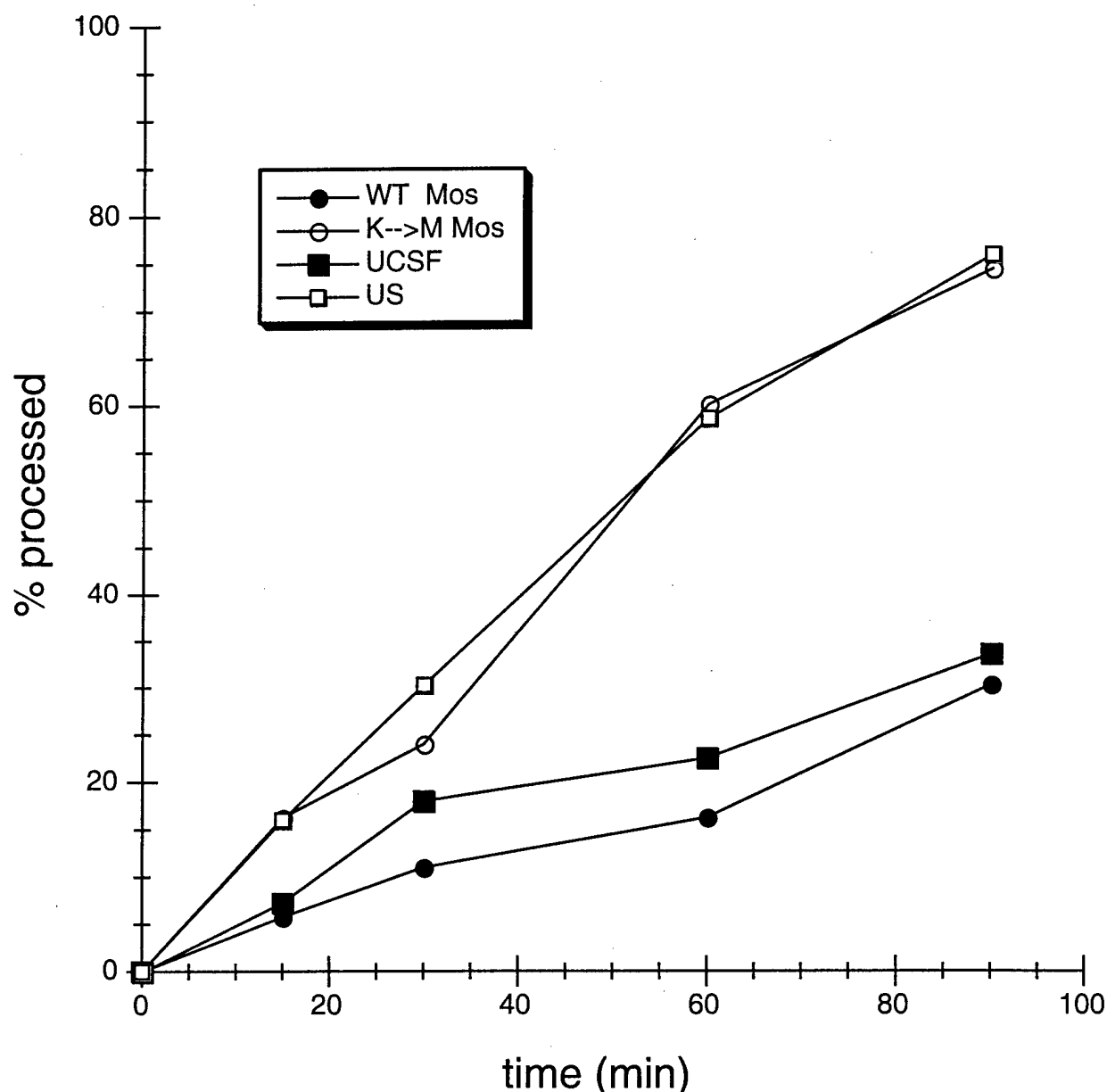
In recent years, it has become increasingly clear that resistance of tumors to chemotheraphy results largely from a failure of apoptosis. The inhibition of apoptosis post-cytochrome c release (which prevents pro-caspase activation and subsequent cell death) may well be a mechanism employed by tumors with a range of activated kinases. We are currently exploring this issue. If, indeed, this

turns out to be the case, then circumventing these protective mechanisms should allow for greater efficacy of chemotherapeutic regmiens.

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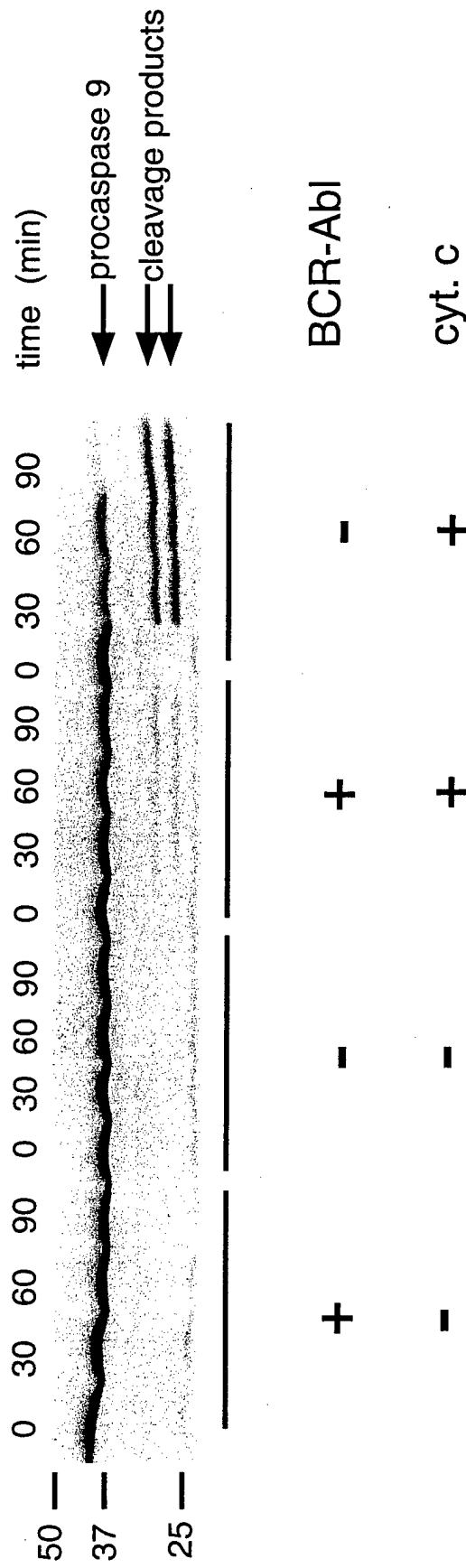
fig1.

MAP kinase activation inhibits the apoptosome

Legend:
- WT Mos
- K-->M Mos
- UCSF
- US

y-axis: % processed (0, 20, 40, 60, 80, 100)
x-axis: time (min) (0, 20, 40, 60, 80, 100)

Apoptosomes were purified from US, UCSF, wild-type Mos-treated US (WT Mos), or kinase dead-treated US (K-->M Mos) on 20 µl cytochrome c beads (Sigma). Purified apoptosomes were then incubated with 5 µl in vitro translated radiolabeled caspase 9 i n 10 ml processing buffer containing 1 mM dATP. Processing reactions were stopped by addition of 15 µl SDS-PAGE sample buffer. Percent caspase 9 processing was assayed by SDS-PAGE and quantitative phosphorimager analysis (Molecular Dynamics).

BCR-Abl Inhibits Caspase 9 Activation

time (min)

50 —
37 —
25 —

0 30 60 90   0 30 60 90   0 30 60 90   0 30 60 90

→ procaspase 9
⇉ cleavage products

BCR-Abl	+	–	+	–
cyt. c	–	–	+	+

Purified Xenopus cytosol containing in vitro-translated radiolabeled caspase 9 was incubated with purified recombinant BCR-Abl or buffer for 30 minutes at room temperature. Cytochrome c was then added to a final concentration 1 µg/40 mg cytosolic protein. Caspase 9 cleavage was analyzed by SDS-PAGE and autoradiography.

# Kinase activity is required for BCR-Abl-mediated inhibition of cytochrome c-dependent caspase 3 activation
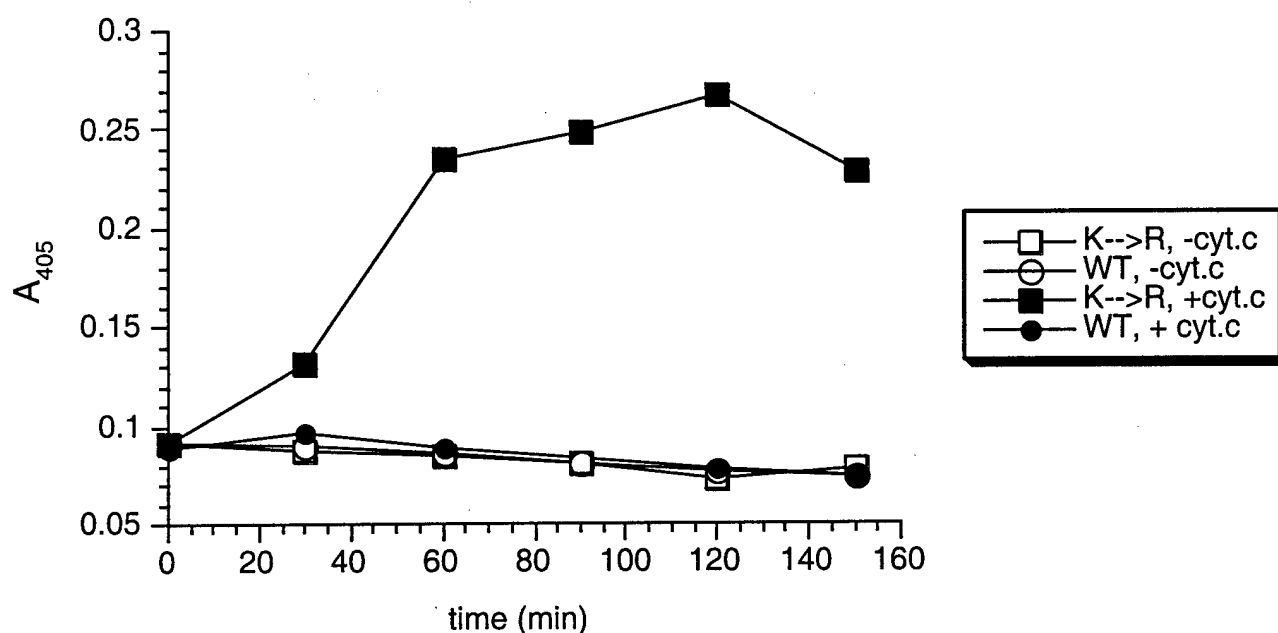
Purified Xenopus cytosol was incubated with biochemically purified wild type BCR-Abl (WT) or kinase dead BCR-Abl (K-->R) for 20 minutes at room temperature. Cytochrome c was then added to 0.9 ng/ml and caspase 3 activity was measured over time using a colorimetric caspase 3 substrate (Biomol).

BCR-Abl does not activate p42 Erk

time (min)

| 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 |

51 —

38 —

p42 Erk (phos)
p42 Erk
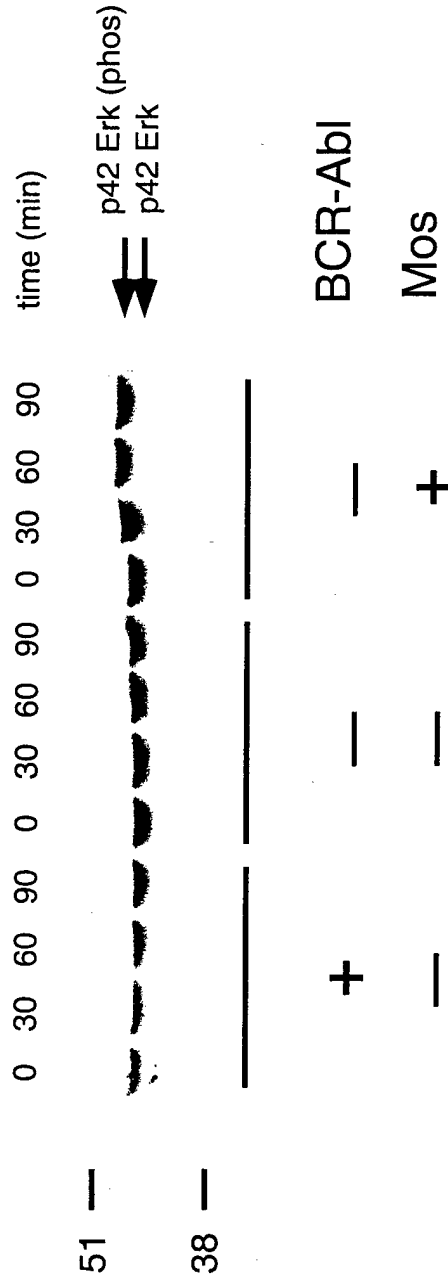
| BCR-Abl | + | — | — |
| Mos | — | — | + |

Purified Xenopus cytosol was incubated with buffer alone or purified recombinant BCR-Abl or Mos. At t = 0, 30, 60, and 90 minutes, 1 µl aliquots were recovered and analyzed for p42 ERK activation by an immunoblot-based gel-shift assay.

Fig. 4.

Post-cytochrome c protection from apoptosis conferred by a

MAPK pathway in Xenopus egg extracts

Jessica S. Tashker, Michael Olson, and Sally Kornbluth*

*To whom correspondence should be addressed:
Dept. of Pharmacology and Cancer Biology
Duke University Medical Center
Box 3813
C370 LSRC, Research Drive
Durham, NC 27710
Phone: 919-613-8624
FAX: 919-681-1005
e-mail: kornb001@mc.duke.edu

Abstract

In response to many different apoptotic stimuli, cytochrome c is released from the intermembrane space of the mitochondria into the cytoplasm, where it serves as a co-factor in the activation of pro-caspase 9. Inhibition of this process can occur either by preventing cytochrome c release or by blocking caspase activation or activity. Experiments involving *in vitro* reconstitution of apoptosis in cell-free extracts of *Xenopus laevis* eggs have suggested that extracts arrested in interphase are susceptible to an endogenous apoptotic program leading to caspase activation, while extracts arrested in meiotic metaphase are not. We report here that Mos/MEK/MAPK pathways active in M phase-arrested eggs are responsible for rendering them refractory to apoptosis. Interestingly, M phase arrested extracts are competent to release cytochrome c, yet still do not activate caspases. Concomitantly, we have also demonstrated that recombinant Mos, MEK and ERK are sufficient to block cytochrome-c-dependent caspase activation in purified *Xenopus* cytosol, which lacks both transcription and translation. These data indicate that the MAP kinase pathway can target and inhibit post-cytochrome c release apoptotic events in the absence of new mRNA/protein synthesis, and that this biochemical pathway is responsible for the apoptotic inhibition observed in meiotic *Xenopus laevis* egg extracts.

Introduction

Apoptosis, or programmed cell death, is the process by which superfluous or damaged cells are removed from the body. Apoptotic pathways are widely conserved and have been studied in organisms ranging from flies and worms to humans. The importance of apoptotic cell death to processes such as developmental body patterning, the immune response to viral infection, and the cellular response to damage cannot be underestimated-- it has been estimated that over 99.9% of the cells generated in the course of a human lifetime die by apoptosis (reviewed in Vaux and Korsmeyer, 1999).

Although a wide variety of stimuli can impinge upon a cell's decision to apoptose, many pro-apoptotic signals converge on the mitochondria, where they promote release of cytochrome c, an integral respiratory chain protein, from the mitochondrial intermembrane space into the cytoplasm (Green and Reed, 1998). Once released, cytochrome c forms a multimeric complex with Apaf-1, a 130 kDa ATP-binding protein (Zou et al., 1999). Thought to stabilize Apaf-1 in its active conformation, cytochrome c renders Apaf-1 competent to recruit the precursor form of one of the "death proteases," caspase 9 (Hu et al., 1999; Jiang and Wang, 2000; Li et al., 1997). Once assembled on the Apaf-1 scaffold, caspase 9 cleaves and activates other pro-caspase 9 molecules within the Apaf-1/caspase 9 complex (Srinivasula et al., 1998). This multimeric complex, containing Apaf-1, cytochrome c, and active caspase 9, is commonly referred to as the

apoptosome (Zou et al., 1999). Once activated within the apoptosome, caspase 9 may then proteolyze and activate other caspases, including caspase 3 (Li et al., 1997), a protease which cleaves a large number of cellular substrates (e.g. nuclear lamins, PARP, the DNAse inhibitor ICAD). These cleavage events are believed to undermine cellular structural integrity and lead to the orderly dismantling of the apoptotic cell (for review see Porter and Janicke, 1999).

Caspase activity is opposed by IAP (Inhibitor of Apoptosis) proteins. IAPs have been shown to bind and potently inhibit many caspases, including caspases –3, -7, and-9, that are known to act downstream of cytochrome c release (Deveraux and Reed, 1999; Deveraux et al., 1998; Roy et al., 1997). Because these IAPs can block cytochrome c-induced caspase activation, they are potent antagonists of cytochrome-c-dependent apoptosis. In turn, IAP function can be antagonized by a diverse group of molecules including the Drosophila proteins HID, GRIM and Reaper (Goyal et al., 2000; Vucic et al., 1997; Vucic et al., 1998), and the human protein SMAC/Diablo. In human cells, SMAC/Diablo binds IAPs and potentiates cytochrome c-dependent caspase 9 processing (Du et al., 2000; Verhagen et al., 2000); therefore, its overexpression increases cellular sensitivity to apoptotic stimuli.

A number of signaling pathways that protect cells from apoptosis appear to block mitochondrial cytochrome c release, which is regulated in an antagonistic fashion by pro and anti-apoptotic members of the Bcl-2 protein family (reviewed in Gross et al., 1999a). When overexpressed, pro-apoptotic

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family members such as Bak and BID potentiate cytochrome c release, while their anti-apoptotic counterparts, Bcl-X$_L$ and Bcl-2, oppose this effect and promote cell survival, either by inhibiting pro-death Bcl-2 family members or by acting directly on mitochondrial components to prevent cytochrome c release (Desagher et al., 1999; Griffiths et al., 1999; Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). Although several reports suggest that apoptosis can also be inhibited *after* the release of cytochrome c from mitochondria (Deshmukh and Johnson, 1998; Erhardt et al., 1999), the signaling pathways effecting such protection, the physiological settings in which this type of cellular protection occurs, and the precise mechanisms of protection have not been clearly defined.

Egg extracts prepared from the frog *Xenopus laevis* provide a useful tool for studying complex cellular processes *in vitro*. Although best known for their use in reconstituting cell cycle processes and nuclear trafficking of macromolecules, these extracts also contain a full complement of apoptotic regulators. Indeed, when egg extracts are "aged" on the bench, they spontaneously recapitulate a range of apoptotic processes, including nuclear fragmentation, DNA laddering, and caspase activation (Newmeyer et al., 1994). These spontaneous apoptotic processes can be blocked by addition of exogenous anti-apoptotic proteins such as Bcl-2 (Evans et al., 1997b), and accelerated by pro-apoptotic proteins such as Bid (Kluck et al., 1999) and the *Drosophila* Reaper protein (Evans et al., 1997b). Although spontaneous apoptosis in egg extracts (as well as apoptosis induced by Bid or Reaper) will not

occur in the absence of mitochondria (Newmeyer et al., 1994), addition of exogenous cytochrome c to fractionated extracts that lack mitochondria results in robust caspase activation (Kluck et al., 1997).

The body of the female frog houses a large pool of immature oocytes, which are arrested in prophase of the cell cycle. In order to stimulate egg production, adult female frogs are subjected to a hormonal regimen that promotes oocyte maturation. Progesterone treatment causes immature oocytes, which are arrested at the start of Meiosis I, to resume progression through the cell cycle while moving down the egg-laying tract, before arresting in metaphase of Meiosis II due to high levels of CSF (Cytostatic Factor) activity (reviewed in Palmer and Nebreda, 2000). Once they are laid, eggs remain arrested in Meiosis II until fertilization, which causes the release of calcium from intracellular stores and induces entry into the cell cycle. Exit from M-phase requires this calcium release, which results in the destruction of both mitotic cyclins and Mos, a MEK kinase that is an integral component of CSF activity and is responsible for activation of the MAP kinase pathway in maturing oocytes and eggs (Sagata et al., 1989; Watanabe et al., 1991).

Although the *Xenopus* egg is arrested in metaphase of Meiosis II, lysis of the eggs by centrifugation while preparing the extracts used in apoptotic reconstitution causes calcium release from internal stores; in the absence of calcium chelators, this release promotes degradation of cyclins and Mos and progression into interphase. The addition of cycloheximide renders the extracts

unable to synthesize new cyclins and therefore unable to re-enter mitosis. Interestingly, Morin and colleagues noted that when eggs are lysed in the presence of calcium chelators in order to preserve their true cell cycle state (Meiotic metaphase), the resulting extracts are markedly refractory to apoptosis. In order to explore the interplay between cell cycle state, signaling pathways, and apoptotic onset, we wished to understand the reason for the differing susceptibility of S phase (interphase) and M phase extracts to undergo apoptosis. We report here that Mos-mediated activation of the ERK MAP kinase pathway, but not Cdc2/Cyclin activity, is necessary and sufficient to render M phase extracts refractory to apoptosis. Strikingly, this MAPK-mediated protection from apoptosis is transcription-independent and occurs predominantly after the release of cytochrome c from mitochondria. Moreover, recombinant Mos, MEK, or ERK proteins are sufficient to block cytochrome-c-dependent caspase activation in purified Xenopus egg cytosol. These results demonstrate that the MAP kinase pathway biochemically targets and inhibits post-cytochrome c apoptotic events in Xenopus eggs.

Material & Methods

Preparation of crude Xenopus egg extracts

To induce egg laying mature female frogs were injected with 100 U pregnant mare serum gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3-10 days later) with human chorionic gonadotropin (hCG) (Sigma). Twenty to twenty-four hours after hCG injection eggs were harvested for extract production. Jelly coats were removed from the eggs by incubation with 2% cysteine pH 8.0, washed three times in modified Ringer solution (MMR) (1.25 mM HEPES pH 7.8, 25 mM NaCl, 0.5 mM KCl, 250 μM MgSO$_4$, 630 μM CaCl$_2$, 28 μM EDTA) and then washed in either ELB (250 mM sucrose, 2.5 mM MgCl$_2$, 50 mM KCl, 10 mM HEPES pH 7.7) for S extract production or in ELB-CSF (ELB + 5mM EGTA pH 8.0) for CSF extract production. Eggs were packed by low speed centrifugation at 400 g. Following addition of aprotinin and leupeptin (final concentration 5 μg/ml), cytocholasin B (final concentration 5 μg/ml) and cycloheximide (50 μg/ml), eggs were lysed by centrifugation at 10,000 g for 15 min.

Fractionation of crude Xenopus egg extracts

To separate mitochondrial and cytosolic components, crude extract was centrifuged at 55,000 r.p.m. (250,000 g) in a Beckman TLS-55 rotor for the TL-100 centrifuge. The cytosolic fraction was removed and re-centrifuged at 55,000

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r.p.m. for an additional 25 min., then aliquotted and frozen in liquid nitrogen for future use. The mitochondrial fraction was diluted 1:1 in MIB (10 mM HEPES pH 7.5, 60 mM sucrose, 210 mM mannitol, 1 mM ADP, 10 mM KCl, 10 mM succinate, 5 mM EGTA) plus 0.5 mM DTT, and then spun through an MIB percoll gradient (42% percoll in MIB, 37% percoll in MIB, 30% percoll in MIB, 25% percoll in MIB). The recovered mitochondrial fraction was washed in MIB and pelleted at 750 g for 10 minutes. The pellet was diluted 1:1 in MIB, then aliquotted and frozen in liquid nitrogen for future use. All extract components were stored at −80° C.

Production of mitochondrial lysates

Frozen mitochondrial pellets were diluted 1:1 in MIB + 25 mM CHAPS on ice for 15 min., then spun through a 0.1 μm ultrafree-MC filter (Millipore) for 15 min at 11,000 r.p.m. in an Eppendorf 5415 C microfuge. The filtrate was collected and protein concentration measured using the BioRad system (BioRad Protein Laboratories).

Immunodepletion assays

For MEK depletion experiments, Protein A-Sepharose beads (Sigma) were washed in PBS and incubated with anti-MEK antibody (kindly provided by Dr. James Ferrell) for 1 hour at 4° C. Bead-antibody complexes were recovered and washed in ELB, then incubated with 100 μl crude extract/ 25 μl beads. After 1

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hour at 4° C the antibody-bead complexes were pelleted and the supernatant transferred to another tube containing more bead-bound antibody. After a second round of immunodepletion the supernatant was collected and supplemented with ATP regenerating system (10 mM phosphocreatine, 2 mM ATP and 150 mg/ml creatine phosphokinase). Extract was then incubated at room temperature and analyzed for caspase 3 activity.

Production of his-tagged proteins

His-MEK R4F and his-MEK kinase dead constructs in the pRSET vector were all kindly provided by Dr. Tom Guadagno. The plasmids were transformed into the BL21DE3 bacterial strain and grown at 37° C for two hours, then induced with 0.4 mM IPTG for four hours. Bacteria were then pelleted at 6,000 X g for 10 min in a Beckman JLA-10.5 rotor, washed in PBS, then re-pelleted. Pellets were frozen in liquid nitrogen and stored at −80° C. For protein production, bacteria were resuspended in 12.5 mls lysis buffer (50 mM HEPES pH 7.7, 750 mM sucrose, 150 mM NaCl, 0.1% Triton X-100)) per liter culture, to which had been added 5 mM β-mercaptoethanol, 1mM PMSF, 5 μM aprotinin and leupeptin, and 0.8 mg/ml lysozyme. Pellets were allowed to lyse on ice for one hour, at which point MgCl$_2$ (final concentration 20 mM), sodium deoxycholate (final concentration 0.15%), and DNAse (0.1 mg total) were added and lysate was left to incubate on ice until no longer viscous, approximately 10-20 minutes. Lysate was centrifuged at 12,000 r.p.m. in a Beckman JS-13.1 rotor for 30 min. Lysate was then poured

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3X over 300 µl Ni-NTA agarose (Qiagen) that had been washed in lysis buffer.

Bead-bound protein was then washed in 15 mls lysis buffer plus 400 mM NaCl

and 20 mM imidazole, then 15 mls lysis buffer alone. For protein elution, beads

were eluted with 5 X 500 µl lysis buffer plus 200 mM imidazole, then the eluate

was concentrated in a Centricon-30 (Amicon), diluted into ELB, then re-

centriconned to the desired volume. Aliquotted proteins were frozen in liquid

nitrogen and stored at −80° C.

Production of MBP-Mos protein

The plasmids pMALcRI-XE and pMALcRI-XE(KM) (Yew et al., 1992) encoding

Xenopus Mos were expressed in the Topp3 bacterial strain, grown two hours at

37° C, then induced with 0.4 mM IPTG for two hours at 37° C. Bacteria were

pelleted at 6,000 X *g* for 10 min in a Beckman JLA-10.5 rotor, washed in PBS,

then re-pelleted. Pellets were frozen in liquid nitrogen and stored at −80° C. For

protein preparation, pellet was resuspended in 25 mls MBP lysis buffer (50 mM

Tris pH 8.0, 50 mM NaCl, 5 mM EDTA) to which had been added 1 mg/ml

lysozyme, 5 µg/ml aprotinin and leupeptin, and 1 mM PMSF. Resuspended

bacteria were then lysed by French press. The lysate was centrifuged at 9,000 *g*

for 20 min. The supernatant was removed and run over Q Sepharose resin that

had been equilibrated with MBP lysis buffer. Salt was added to the flow-through

to reach a final concentration of 0.5 M NaCl. The flow-through was passed twice

over an amylose resin, which was then washed with amylose column buffer (20

mM HEPES pH 6.8, 88 mM NaCl, 7.5 mM MgCl$_2$) plus 410 mM NaCl, then with

amylose column buffer alone. Protein was eluted with 10 X 1 ml fractions of

amylose column buffer plus 10 mM maltose. The fractions with the highest

protein concentration as measured by A$_{280}$ were pooled and concentrated using

PEG (Sigma), then dialyzed overnight in ELB. Protein was aliquotted and frozen

in liquid nitrogen, then stored at –80° C..

Caspase 3 Activity Assays

To measure caspase 3 activity, 3 μl of each sample was incubated with 10 μl

colorimetric substrate AC-DEVD-pNA (Biomol) in Assay Buffer (50 mM HEPES

pH 7.7, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, and 10 mM

DTT) for 1 hour at 37°C. After 1 hour incubation the reaction was stopped by the

addition of 0.2 μM Ac-DEVD-CHO (Biomol). Reaction was read at 405 nm with a

Labsystems Multiscan Plus plate reader (Fisher Scientific).

Cytochrome c release assays

For assays in crude extracts the extract was supplemented with ATP

regenerating mixture. At various time points cytosolic cytochrome c content was

analyzed by diluting 15 μl crude extract into 15 μl ELB and filtering diluted extract

through a 0.1 μm ultrafree-MC filter (Millipore). The filtrate was run on 17.5%

SDS-PAGE minigels and blotted with anti-cytochrome c antibody (Pharmingen,

Cat# 556433).

ERK thiophosphorylation

Recombinant ERK1 (Cat #14-188, Upstate Biotechnology) was thiophosphorylated by diluting 12.5 μl enzyme (stored in PBS + 50% glycerol) 1:1 with 2X thiophosphorylation buffer (40 mM Tris pH7.5, 40 mM MgCl$_2$, 0.2 mM EDTA, 30 mM β-mercaptoethanol, and 1 mM ATP-γ-S) and incubating with his- MEK immobilized on nickel beads for 4 hours at 30°C. As a control, the reaction was also carried out with PBS + 50% glycerol not containing any enzyme. The beads were centrifuged to remove the MEK kinase and the thiophosphorylated ERK was collected. In order to remove residual ATP-γ-S, the activated enzyme was diluted out to 500 μl in ELB and passed through a Microcon YM-10 (Millipore Cat # 42406) until greater than 10-fold re-concentration was achieved; this step was repeated three times. ERK activity was measured using recombinant MBP as a substrate.

Results

M phase extracts are resistant to apoptosis

As described above, it has been observed that interphase egg extracts are considerably more susceptible to apoptosis than are extracts prepared so as to preserve the meiotic arrest of the egg. In order to verify this observation, we wished to compare spontaneous apoptotic activity in extracts stably arrested in M phase (hereafter referred to as CSF extracts, for "Cytostatic Factor-arrested") and interphase (S) extracts, as well as in CSF extracts that had been released into interphase by addition of exogenous calcium (CSF + Ca^{2+}). In order to exclude the possibility that apoptotic inhibition was due to artificial sequestration of calcium by the chelating agent used during CSF extract preparation, EGTA was also added to S extracts (S + EGTA) after Ca^{2+}-induced release into interphase; these extracts are unable to return to an M phase state as mitotic cyclins are not present.

As an apoptotic marker, we chose to evaluate caspase activity as measured by cleavage of the model caspase substrate AC-DEVD-pNA; cleavage of the substrate results in a product that can be monitored spectrophotometrically at 405 nm. In a typical experiment, both types of interphase extract (S and S + EGTA) developed caspase activity at hour three of a six-hour incubation, while the released CSF extract (CSF + Ca^{2+}) was slightly delayed in apoptotic

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activation, exhibiting robust caspase activity after 4 hours. Of all the extracts tested, only stably-arrested CSF extracts showed no spontaneous caspase activity over the time course observed (Fig 1). This experiment confirms the observation that CSF extracts are resistant to apoptosis, and also demonstrates that this resistance is due to properties of the meiotic CSF extract, rather than to non-specific effects of the chelating agent.

CSF extracts are resistant to cytochrome c-induced caspase activation

In many cell types, mitochondria serve as a repository of pro-apoptotic components that are released into the cytosol upon receipt of apoptotic stimuli (reviewed in Earnshaw, 1999). As reported by Newmeyer and colleagues (Newmeyer et al., 1994), apoptosis in the *Xenopus* extract system is absolutely dependent upon a heavy membrane fraction containing mitochondria, implying that mitochondrial factors are required to generate spontaneous caspase activity in the extract. Given this observation, we hypothesized that CSF and interphase extracts might differ either in their propensity to release mitochondrial factors or in their susceptibility to the pro-apoptotic influence of such factors once released.

As a marker for release of mitochondrial contents, we elected to monitor efflux of cytochrome c, the only apoptotic regulator known to reside in the mitochondrial intermembrane space thus far well-characterized in the *Xenopus* system. At various time points after initiating room temperature incubation, we

passed M phase (CSF) and interphase (CSF + Ca^{2+}) extracts through 0.1 μm

filters in order to exclude all intact organelles, including mitochondria. The filtrate,

which contains cytosolic components but lacks mitochondria, was then assayed

for the presence of cytochrome c by SDS-PAGE and immunoblotting with anti-

cytochrome c antibodies. Interestingly, both the interphase (CSF + Ca^{2+}) and M

phase (CSF) extracts showed robust cytochrome c release, although the CSF

extract lagged slightly behind the interphase extract (Fig. 2A). However, by 4

hours, the mitochondria within the CSF extract had released considerable

quantities of cytochrome c, yet did not, even by hour 7 of the experiment, activate

caspases (Fig 2B). From these data we concluded that CSF phase extracts are

quite capable of inducing cytochrome c release, yet still do not activate

caspases. Although the slight lag in cytochrome c release compared to S

extracts indicates that factors within the CSF extract may retard release of

mitochondrial components, our data strongly indicate that CSF extracts also

contain potent factors that can prevent caspase activation downstream of

mitochondrial cytochrome c release. These factors appear to be either lacking or

less active in interphase extracts.

To demonstrate unequivocally the differential sensitivity of these extracts

to mitochondrial contents as a whole (which contain not only cytochrome c, but

presumably homologues of other apoptotic regulators such as SMAC/Diablo and

AIF), we separated mitochondrial and cytosolic fractions from crude extract by

centrifugation, then lysed the mitochondria in a detergent-containing buffer and

recombined this lysate with purified cytosol derived from either the S or CSF extracts (US or UCSF , for ultra-centrifuged S or CSF). These reconstituted extracts were then incubated at room temperature and monitored for the development of caspase activity. As shown in Fig. 3A, the CSF cytosol was markedly refractory to induction of caspase activity by total mitochondrial protein, although excess mitochondrial protein could overcome this resistance (our unpublished results). In contrast, S cytosol was fully susceptible to caspase activation even by low concentrations of mitochondrial protein. These results indicate that CSF and S phase extracts are differentially sensitive to pro-apoptotic factors present in the mitochondria, and that cytosolic factors present in CSF extracts can protect extracts from these pro-apoptotic factors.

It has been shown that exogenous cytochrome c is sufficient to activate caspases 9 and 3 in purified cytosol (Kluck et al, 1997; Li et al, 1997). Because we had demonstrated that cytochrome c is released from mitochondria in CSF extracts and that CSF extracts are relatively insensitive to the pro-apoptotic influence of mitochondrial contents, we wished to determine whether factors within CSF extracts could prevent caspase activation by pure cytochrome c. Therefore, we added purified cytochrome c (Sigma) to S or CSF cytosol (lacking mitochondria) and monitored caspase activity. As shown in Fig 3B, when compared to interphase cytosols, CSF cytosols were markedly resistant to cytochrome c-induced caspase activation.

Mos/MEK kinase pathway activity is necessary and sufficient for apoptotic inhibition in M phase extracts

The most notable difference between CSF and interphase extracts is the presence of high levels of mitotic Cyclin/Cdk activity in the former. Indeed, during conversion of mitotic extracts to interphase extracts (or during lysis of eggs in the absence of calcium chelators), the mitotic cdk cdc2/cyclin B is inactivated by calcium-dependent destruction of cyclin B (Watanabe et al., 1991). We therefore assumed that the difference between M and S extracts might lie in the differing levels of cdc2/cyclin B activity. In support of this notion, we found that addition of recombinant cyclin B to interphase extracts could prevent the development of caspase activity (Fig. 4A). We were surprised, therefore, when the drug roscovitine, a potent inhibitor of cdc2/cyclin B activity, was unable to promote apoptosis in CSF extracts (our unpublished results). However, because the addition of Cyclin B to interphase extracts also activates the MEK/ MAP kinase pathway (Guadagno and Ferrell, 1998), which is also highly active in CSF extracts, we hypothesized that a MAP kinase pathway might be responsible for the observed apoptotic inhibition.

The MEK kinase Mos, together with cyclin B, is a primary target of calcium-dependent destruction during the transition from M phase to interphase in the *Xenopus* system (Watanabe et al., 1991). Based on our previous results, we hypothesized that continued stimulation of Mos/MEK kinases might be required to block apoptosis in CSF extracts. In order to test this hypothesis, we

immunodepleted endogenous MEK from crude CSF extracts using an anti-MEK antibody. As shown in Fig. 4B, immunodepletion of CSF extracts with MEK antibodies, but not control IgG, restored apoptotic activity, indicating that MEK, and, by extension, its activator, Mos, are required to maintain apoptotic inhibition in CSF extracts.

In order to determine if Mos activation of MEK was sufficient to recapitulate the post-cytochrome c protection from apoptosis observed in CSF extracts, we incubated interphase cytosol with recombinant tagged wild-type Mos (WT Mos) or kinase-inactive Mos (K→M Mos), then added recombinant cytochrome c. Mos kinase activity was sufficient to block caspase activity in the presence of cytochrome c (Fig 5A). This effect was completely reversed by UO126, a MEK inhibitor, indicating that Mos-dependent inhibition of cytochrome c-mediated caspase 3 activity is, as anticipated, mediated through MEK.

We extended these findings by incubating interphase cytosol with recombinant constitutively active (R4F) or kinase-dead (KD) MEK, then adding exogenous cytochrome c. R4F MEK alone, but not its kinase inactive variant, was sufficient to block cytochrome c-induced caspase activity (Fig 5B). Collectively, these data indicate that MEK activity is sufficient to maintain apoptotic inhibition in mitotic extracts, and suggest that this inhibition is, at least in part, directed at post-cytochrome c apoptotic events.

ERK is sufficient to block cytochrome c-dependent caspase activation

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Because our experiments demonstrated that MEK was able to block cytochrome c-dependent caspase activation, we wished to determine whether this anti-apoptotic effect was exerted through its target kinase, the serine/threonine kinase MAP kinase, ERK. As there are no ERK-activating stimuli in the interphase extract, it was first necessary to activate purified wild-type ERK protein using recombinant MEK. To render the activated ERK resistant to inactivating phosphatases present in the extract, we carried out this phosphorylation in a reducing buffer in the presence ATP-γ-S (Haccard et al., 1993). As shown in Figure 6, interphase egg cytosol supplemented with this activated ERK preparation was resistant to cytochrome c-induced caspase activation. Collectively, these data demonstrate that the Mos-MEK-ERK pathway can target and inhibit post-cytochrome c apoptotic events. Moreover, the resistance of CSF extracts to cytochrome c results from the constitutive activation of this pathway in Xenopus eggs.

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Discussion

Egg extracts prepared from the frog *Xenopus laevis* can initiate and execute a full apoptotic program *in vitro*. We have used this biochemically tractable system to demonstrate that the Mos/MEK/MAP kinase pathway is necessary and sufficient to inhibit *in vitro* apoptotic processes, and that this inhibition is directed at a post-cytochrome c release, pre-caspase activation step. Since *Xenopus* egg extracts lack pol II transcription and are prepared in the presence of cycloheximide, this surprising result indicates that the MAP kinase molecule ERK can prevent cytochrome c-dependent caspase activation in the absence of transcription or protein synthesis.

Post-cytochrome c protection from apoptosis

Our results, along with those of Morin and colleagues, demonstrate that CSF extracts, made so as to preserve the meiotic arrest of the intact egg, are refractory to the *in vitro* apoptotic program initiated in interphase egg extracts (Faure et al., 1997). Interestingly, we have shown that CSF extracts release cytochrome c, albeit with slightly delayed kinetics when compared with interphase extracts, and yet remain resistant to caspase activation. This result suggests that some component of CSF extracts can inhibit caspase activation at a step downstream from cytochrome c release; we have demonstrated that the required factor(s) is an activated MAP kinase pathway.

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The ability of MAP kinase pathways to promote cell survival has been well-documented (Bonni et al., 1999; Hetman et al., 1999; Holmstrom et al., 2000). However, because of the limitations imposed by tissue culture systems, it has not been feasible to separate out transcription-dependent and –independent effects of MAP kinase activation on post-cytochrome c events. For example, Cooper and colleagues (Erhardt et al., 1999) have shown that lysates from Rat-1 cells transfected with B-Raf, an upstream activator of MAP kinases, are resistant to cytochrome c-induced caspase activation. They have proposed that MAP kinase pathway activation may result in increased expression of anti-apoptotic molecules, such as IAPs, that can inhibit caspase activation downstream from cytochrome c release. However, because our system does not support transcription or translation, our data demonstrate the existence of a more direct biochemical role for the MAP kinase pathway in preventing caspase activation after release of cytochrome c from the mitochondria.

The existence of a MAPK-mediated mechanism to prevent apoptosis after cytochrome c release begs the question as to why cells would inhibit these processes when the more upstream event, release of cytochrome c from the mitochondria, is so thoroughly regulated. The simple answer may be that apoptosis, like any other cellular process, is regulated at multiple steps so as to prevent cells from making the "wrong" decision, and that multiple negative regulatory events help to protect the cell in case of accidental damage. A more complex answer may involve examining the types of cells in which this protection

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can be observed. For example, Deshmukh and Johnson (Deshmukh and Johnson, 1998) have demonstrated that sympathetic neurons are insensitive to microinjected cytochrome c in the presence of growth factors; they propose that a high level of resistance to cytochrome-c induced apoptosis may be necessary for cells such as post-mitotic neurons, which are not easily replaced. In *Xenopus*, the apoptosis-resistant meiotic stage of the cell cycle corresponds to eggs that are en route to being laid or have already been laid. Although these cells can easily be replaced, allowing gametes to apoptose is disadvantageous for organisms such as frogs, which have a low energy investment in their offspring and hence are advantaged by producing the largest possible number of gametes available for fertilization. Alternatively, it may be that apoptotic inhibition is simply a by-product of the high level of Mos/MEK/MAP kinase activity required to maintain the metaphase II meiotic arrest. In somatic cells, this degree of MAP kinase activation would be observed only after particular signaling events, while in the egg this pathway is, by necessity, constitutively active.

Since interphase egg extracts, which no longer have high levels of MAP kinase activity, do not spontaneously release cytochrome c until they have been incubated at room temperature for prolonged periods, we assume that there are apoptotic inhibitors operating prior to cytochrome c release in these extracts (and most likely, in the early fertilized embyros that they mimic.) Indeed, it has been suggested that apoptosis is suppressed during the early cleavages in the Xenopus embryo (pre-mid blastula transition) by maternally encoded apoptotic

inhibitors (Stack and Newport, 1997; Hensey and Gautier, 1997). While post cytochrome c protection conferred by MAPK is likely to be lost at fertilization, other, pre-cytochrome c release mechanisms must act to prevent apoptosis during the early embryonic cleavages.

MAP kinase and the apoptosome

Once released into the cytosol, the primary function of cytochrome c is to nucleate the apoptosome through recruitment of Apaf-1 and caspase 9. Because our data indicates that MAP kinase targets a post-cytochrome c event, it seems likely that the MAP kinase pathway might target and modulate this initial downstream event, the formation or function of the apoptosome. In theory, apoptosomal inhibition could result from a change in the composition of the apoptosome or from the post-translational modification (i.e. phosphorylation) of pre-existing components. However, the possible targets are not limited to Apaf-1, caspase 9, and cytochrome c; a comparison of apoptosomes isolated from cell lysates with *in vitro* reconstitutions using purified recombinant components (i.e. caspase 9, cytochrome c, Apaf-1 and dATP) have suggested that apoptosomes from cell lysates may contain additional factors (Cain et al., 2000). Moreover, a number of accessory proteins associated with apoptosomes have been described (e.g. Aven (Chau et al., 2000) and NAC (Chu et al., 2001)). The function of these or other novel molecules may be altered by MAP kinase phosphorylation; further investigation will concentrate on identifying the relevant

MAP kinase target(s). Another possibly relevant MAP kinase target is HID, a known *Drosophila* IAP inhibitor. Because HID has been shown to be a MAP kinase substrate in flies (Bergmann et al., 1998), it is attractive to speculate that apoptosomal association of IAPs might be altered in response to the activity of a HID-like protein in egg extracts.

In aggregate, our data both explain the relative resistance of meiotic extracts to apoptosis and describe the pathway responsible for this phenomenon. The inhibition of cytochrome c-dependent caspase activation by ERK, coupled with our use of a transcriptionally/translationally inert system, demonstrates unequivocally the existence of a purely post-translational inhibition of apoptosis by MAP kinase pathways. Moreover, a good deal of this inhibition appears to occur after mitochondrial release of cytochrome c, providing a novel context in which this type of inhibition can be observed. Finally, these findings offer a starting point for future identification of possible MAP kinase-modified apoptotic regulators, including apoptosomal components, IAPs and their regulators, and other proteins acting downstream of mitochondrial cytochrome c release.

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Figure Legends

Figure 1. CSF extracts do not undergo apoptotic caspase activation. Crude interphase (S), meiotic (CSF), and CSF extracts that had been supplemented with exogenous calcium in order to drive them into interphase (CSF + Ca^{2+})were analyzed for caspase activity at various time points using the model caspase substrate AC-DEVD-pNA. As a control, EGTA was added to S extracts after lysis-induced entry into interphase. Cleavage of the caspase substrate AC-DEVD-pNA was measured spectrophotometrically at 405 nm (see methods).

Figure 2. CSF extracts release cytochrome c but do not activate caspases. (A) Crude CSF extract and CSF extract that had been supplemented with exogenous calcium was analyzed for cytochrome c release at various time points by filtering aliquots of extract through a 0.1 μm microfilter. The filtrate, which lacks mitochondria, was analyzed by SDS-PAGE and Western blotting using an anti-cytochrome c antibody. (B) Crude untreated CSF extract and CSF extract that had been supplemented with exogenous calcium was assayed for caspase activity as described for Fig. 1.

Figure 3. CSF cytosol is resistant to caspase activation. Crude S and CSF extract were further fractionated into membranous and cytosolic fractions. (A) Mitochondrial fractions that had been purified away from the membranous

fraction by centrifugation through a percoll gradient were then lysed in a buffer containing detergent. The lysate was then filtered through a 0.1 μm microfilter. The filtrate was then added to S or CSF cytosol (US or UCSF, respectively) to a final concentration of 1 mg mitochondrial protein per 60 mg extract protein. Caspase activity was measured as described for Fig. 1. Cytochrome c is required for caspase activation by mitochondrial lysate. (B) Purified cytochrome c was added to US or UCSF to a final concentration of 1 μg cytochrome c per 40 mg extract protein. Caspase activity was measured as described in Fig. 1.

Figure 4. Depletion of MEK promotes apoptosis in crude CSF extracts. (A) Buffer or recombinant cyclin B was added to crude S extract and apoptosis was measured using an AC-DEVD-pNA cleavage assay. (B) MEK was immunodepleted with purified anti-MEK antibody (see methods) or mock-depleted with purified IgG from 100 μl crude extract in two successive rounds of depletion at 4° C. Immunodepletion was assayed using SDS-PAGE and Western blotting; 95-99% depletion was achieved (our unpublished results). After immunodepletion, extracts were incubated at room temperature and caspase activity was measured at various time points as described in Fig. 1. Note that immunodepletion of the extract with either the control or anti-Mek IgG delays apoptotic onset somewhat; this effect is most likely due to dilution of the extract during the immunodepletion process.

Figure 5. Mos and MEK prevent cytochrome c-dependent caspase activation. (A) Interphase cytosol (US) was treated for one hour at room temperature with recombinant tagged wild-type Mos (WT Mos) or with kinase dead Mos (K→M Mos), in the presence or absence of 50 μM UO126, a MEK inhibitor. Purified cytochrome c was then added to US or UCSF to a final concentration of 1 μg cytochrome c per 40 mg extract protein. Caspase activity was measured using an AC-DEVD-pNA cleavage assay. (B) Interphase cytosol was treated with recombinant tagged constitutively active MEK (R4F MEK) or kinase dead MEK (KD MEK) for one hour at room temperature. Cytochrome c was then added to 1 μg per 40 mg extract protein. Caspase activity was measured as described for Fig. 1.

Figure 6. Thiophosphorylated ERK inhibits cytochrome c-dependent caspase activation. Interphase cytosol was treated with thiophosphorylated ERK or equivalently prepared buffer (see methods) for 30 minutes at room temperature. Cytochrome c was then added to 1 μg per 40 mg extract protein. Caspase activity was measured as described for Fig. 1.
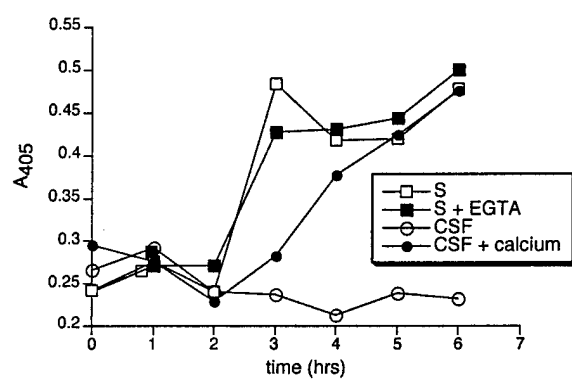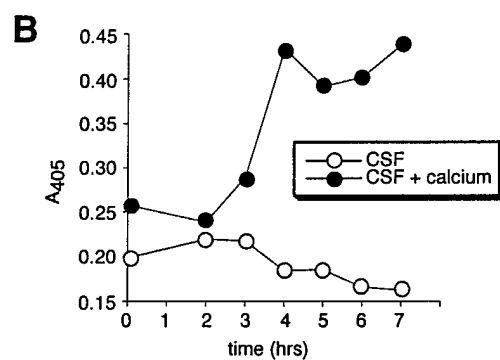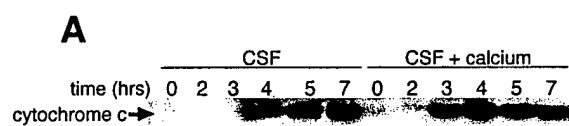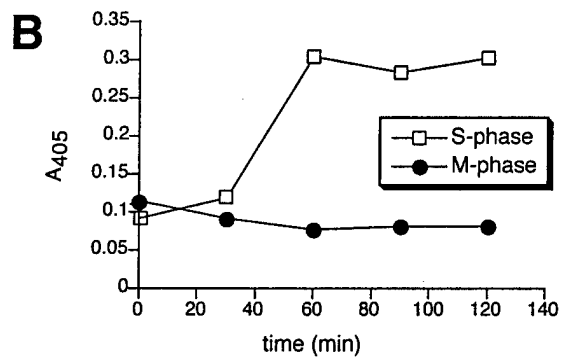
35

Fig 1.

Fig 2

A

	CSF						CSF + calcium					
time (hrs)	0	2	3	4	5	7	0	2	3	4	5	7

cytochrome c ➤

B

Fig 3

Fig 4

A

B
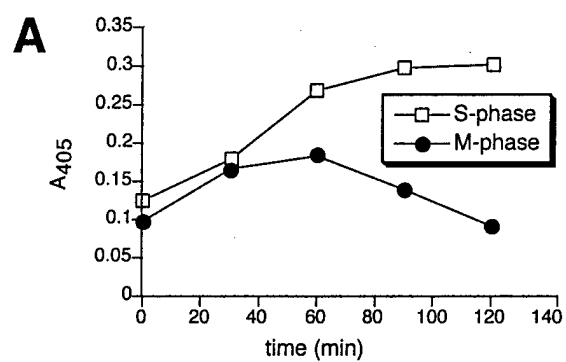
Fig 5

Fig 6
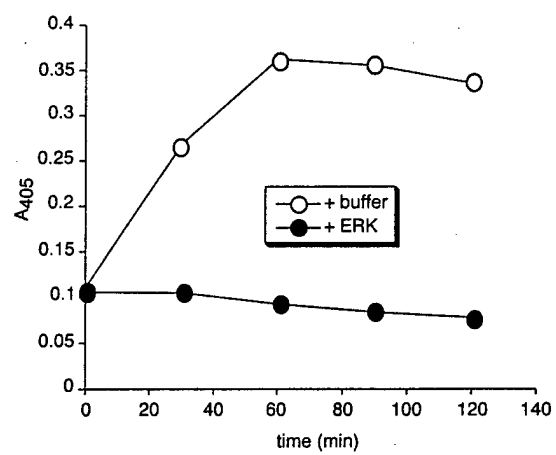
Apoptotic regulation by the Crk adaptor protein

mediated by interactions with Wee1 and Crm1/exportin

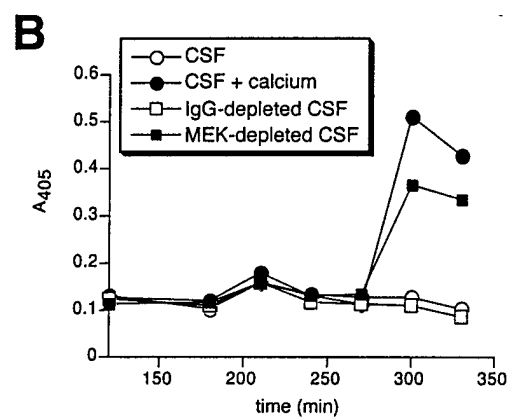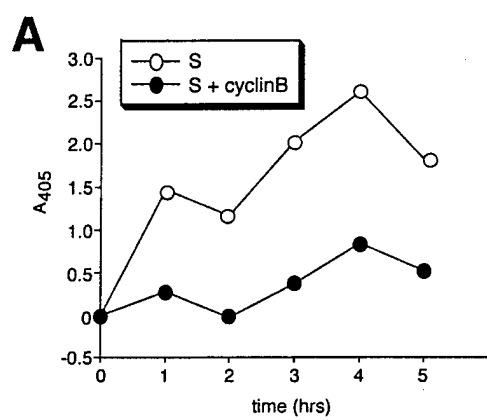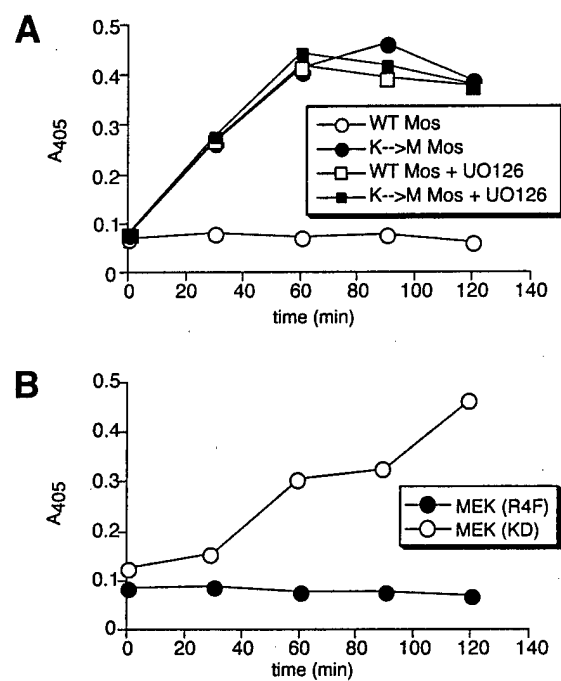Jesse J. Smith[1], D. Ashley Richardson[1], Jan Kopf[2],
Minoru Yoshida[3], Robert E. Hollingsworth[2], and Sally Kornbluth[1]*

*To whom correspondence should be addressed:

[1]Dept. of Pharmacology and Cancer Biology
Duke University Medical Center
Box 3813, C370 LSRC, Research Drive
Durham, NC 27710
Phone: 919-613-8624
FAX: 919-681-1005
Email: kornb001@mc.duke.edu

[2]Pathway Discovery
GlaxoSmithKline, Inc.
5 Moore Drive
Research Triangle Park, NC 27709

[3]Department of Biotechnology
Graduate School of Agriculture & Life Sciences
The University of Tokyo
Yayoi 1-1-1, Bunkyo-ku
Tokyo 113-8657, Japan

Abstract

The adaptor protein Crk contains an SH2 domain and two SH3 domains. Through binding of particular ligands to the SH2 domain and the N-terminal SH3 domain, Crk has been implicated in a number of signaling processes including regulation of cell growth, cell motility, and apoptosis. We report here that the C-terminal SH3 domain, never shown to bind any specific signaling molecules, contains a binding site for the nuclear export factor, Crm1. We find that a mutant Crk protein, deficient in Crm1 binding, promotes apoptosis. Moreover, this nuclear export sequence mutant (NES(-) Crk) interacts strongly, through its SH2 domain, with the nuclear tyrosine kinase, Wee1. Collectively, these data suggest that a nuclear population of Crk bound to Wee1 promotes apoptotic death of mammalian cells.

Introduction

In many different intracellular signaling pathways, information is transmitted through protein-protein interactions mediated by the modular protein binding domains, SH2 and SH3 (for review, Pawson and Gish 1992; Birge et al. 1996). These domains, which bind tyrosine phosphorylated proteins and polyproline-rich motifs, respectively, can be contained either within large enzymes, such as c-Abl, where they may regulate catalytic activity, substrate selection and interaction with upstream regulators, or within small adaptor proteins, such Crk, Nck, and Grb2, which contain no intrinsic enzymatic activity.

The predominant paradigm for adaptor protein signaling involves localization of adaptor-bound SH3 ligands to specific subcellular locales via interaction of the SH2 domain of the adaptor with specific tyrosine phosphorylated proteins. This is exemplified by the localization of the Ras GTP exchange factor, Sos, to the plasma membrane following ligand engagement of receptor tyrosine kinases (e.g. epidermal growth factor receptor; Bowtell et al. 1992). Through binding to tyrosine phosphorylated residues on the intracellular domain of the receptor, the adaptor protein Grb2 brings SH3-bound Sos to the membrane where it can activate Ras (Buday and Downward 1993; Chardin et al. 1993; Rozakis-Adcock et al. 1993).

That inappropriate adaptor protein signaling can have severe consequences for the cell was first suggested by the observation that a protein with homology to the viral oncoprotein Src, but lacking any obvious catalytic domain, could promote oncogenic transformation (Mayer et al. 1988; Tsuchie et al. 1989). This protein, v-Crk, encoded by

the avian sarcoma virus, CT10, contains the viral Gag protein fused to sequences encoding an SH2 domain and an SH3 domain. Two cellular homologs of this protein, Crk-I and Crk II, have since been shown to consist of one SH2 domain and either one or two SH3 domains, respectively (Matsuda et al. 1992; Reichman et al. 1992; for review Matsuda and Kurata 1996; Feller et al. 1998). The Crk-II protein, containing two SH3 domains, is at least 10-fold more abundant than Crk I in most tissues and the linker region between the Crk-II SH3 domains contains a site of potential tyrosine phosphorylation, believed to serve as a site of regulatory intramolecular SH2 binding (Reichman et al. 1992; Feller et al. 1994; Escalante et al. 2000). Finally, a close relative of Crk (CrkL) has been identified that has overall structural similarity and high sequence homology to Crk II (ten Hoeve et al. 1993; Nichols et al. 1994; Oda et al. 1994).

Since Crk lacks intrinsic catalytic activity, a good deal of effort has gone into identifying binding partners for its SH domains and determining the physiological contexts in which they act. Crk has been linked to cell proliferation through its SH2-mediated interactions with tyrosine phosphorylated Cbl, Shc and EGF receptor (Birge et al. 1992; Matsuda et al. 1994; Buday et al. 1996; for review, Feller et al. 1998). More recently, it has become clear that Crk plays a role in cell adhesion signaling and actin reorganization through Crk recruitment of SH3-bound Dock 180 (a regulator of the GTPase Rac) to tyrosine phosphorylated p130Cas, found at focal adhesions and sites of membrane ruffling (Harte et al. 1996; Hasegawa et al. 1996; Kiyokawa et al. 1998; Klemke et al. 1998; Cheresh et al. 1999; Cho and Klemke 2000). Additionally, using

cell-free extracts prepared from Xenopus eggs, we have previously implicated Crk in apoptotic signaling (Evans et al. 1997b; Smith et al. 2000).

Although Xenopus egg extracts are best known for their use in reconstituting cell cycle progression and nuclear trafficking, more recently it was shown that these extracts can be used to examine the morphological and biochemical events of apoptosis (Newmeyer et al. 1994; Evans et al. 1997a; Evans et al. 1997b; Kluck et al. 1997a; Kluck et al. 1997b; Thress et al. 1998; Smith et al. 2000). As is the case in most intact mammalian cells, apoptosis in these extracts is characterized by activation of apoptotic proteases (caspases), release of cytochrome c from the intermembrane space of the mitochondria to the cytosol (where it serves as a co-factor in caspase 9 activation), activation of Dnases, and concomitant fragmentation of nuclei. Importantly, these hallmarks of apoptosis, that appear after extended room temperature incubation of the extract, can be prevented by common inhibitors of apoptosis such as ZVAD, YVAD, DEVD (caspase inhibitors) and anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL (Newmeyer et al. 1994; Evans et al. 1997a; Kluck et al. 1997a; Kluck et al. 1997b).

When we analyzed the requirements for apoptosis in Xenopus extracts, we found that the adaptor protein Crk was absolutely required for mitochondrial cytochrome c release and consequent caspase activation (Evans et al. 1997b). Indeed, immunodepletion of endogenous Crk protein or addition of anti-Crk sera to the extracts completely abrogated apoptotic signaling. Perhaps most surprising was our finding that the Crk SH2 ligand important for pro-apoptotic signal transmission in these extracts was

the known Cdc2/Cyclin B inhibitor, Wee1 (Smith et al. 2000). In a series of biochemical experiments, we demonstrated that Wee1, like Crk is required for apoptotic activation of Xenopus egg extracts. Furthermore, Wee1's pro-apoptotic function depends upon its interaction with Crk. Because chemical inhibitors of Cdc2 as well as the Wee1 related Cdc2/Cyclin regulator, Myt1, did not exhibit apoptotic effects similar to that of Wee1, we hypothesized that the role of Wee1 in apoptosis is distinct from its cell cycle regulatory role and involves signaling via the Crk adaptor protein (Smith et al. 2000).

We report here the finding that mammalian Crk-II is similar to its Xenopus counterpart in its ability to bind tyrosine phosphorylated Wee1. In searching for other potential components of this complex, we also made the surprising discovery that the second SH3 domain of Crk-II, which, unlike the first SH3 domain, has never been shown to bind any signaling molecule, interacts with the nuclear export receptor, Crm1. We have identified a candidate Crm1-binding nuclear export sequence (NES) within the second (C-terminal) SH3 domain and show that ablation of this sequence both enhances Wee1/Crk binding and increases the pro-apoptotic activity of the Crk protein. These findings implicate a nuclear pool of Crk/Wee1 complexes in signaling pathways that promote apoptosis.

Materials and Methods:

Plasmids and Primers:

pCDNA 3.0 myc-Wee1 was graciously provided by Helen Piwnica-Worms, (Department of Cell Biology and Physiology, Washington University, School of Medicine, St. Louis, MO). pCNDA 3.0-caspase-8 was the generous gift of Vishva Dixit (Department of Molecular Oncology, Genentech Inc., San Francisco, CA). GST fusions of Crk SH2 domain, SH3(N) domain, and SH3(C) domain constructs have been described previously (Evans et al. 1997b). The GST fusion of the cyclin B1 NES (nuclear export sequence, or cytoplasmic retention sequence) has been described previously (Yang et al. 1998). wt Crk II cloned into the vector pEBB has been described previously (Tanaka et al. 1995) and was provided by Dr. Bruce J. Mayer (Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut). NES(-) Crk cloned into pEBB was generated by overlap extension using the polymerase chain reaction (Ho et al. 1989). The PCR product (NES(-) Crk) was cloned into pEBB using BamHI and NotI sites at the 5' and 3' ends respectively. The mutagenesis primers used for this procedure are as follows:

INNER PRIMERS

(5'-TTGGAGGTCGGTGAGGCGGCAAAGGCCACGAAGATTAACATGAGT-3'

5'-ACTCATGTTAATCTTCGTGGCCTTTGCCGCCTCACCGACCTCCAA-3')

OUTER PRIMERS

5'-GGATCCACCATGGCCGGGCAG-3'

5'GCGGCCGCTCAGCTGAAGTC-3'

pEBB-myc-wt Crk and pEBB-myc-NES(-) Crk by inserting a 3Xmyc tag at the 5' end of the Crk constructs using the BamHI site. GST-NES(-) Crk SH3(C) construct was prepared by PCR amplification of the SH3(C) domain of pEBB NES(-) Crk. The PCR product was cloned into pGex kg (a derivative of pGex 2T, Pharmacia) using BamHI and HindIII sites at the 5' and 3' ends respectively. Primers used for this process were as follows:

5'-GGATCCTTGGCTTTGGAGGTCGGTGAG-3'

5'-AAGCTTTCATTGATCCAGCAGGCGGACATG-3'

For expression of GST-wt Crk and GST-NES(-) Crk in bacteria, the respective cDNAs were excised from pEBB using BamHI and XbaI (5' and 3' ends respectively) and subsequently cloned into pGex kg. Expression and purification of GST fusion proteins in bacteria was performed according to Evans et al. 1997b.

Yeast two-hybrid

The cDNA insert from pEBB wt CrkII was PCR amplified and cloned into the DNA binding domain fusion vector PMW101, which has been described previously (Watson et al. 1996). Yeast two-hybrid screening protocol was performed as has been described previously (Slentz-Kesler et al. 2000). The Crk II bait was screened versus one million clones derived from human fetal brain, human fetal liver, and human testis prey libraries (purchased from InVitrogen, Inc.).

Antibodies:

Anti-myc monoclonal antibody (clone 9E10) was purchased from Santa Cruz. Anti-FLAG monoclonal antibody was obtained from Sigma. Anti-phosphotyrosine monoclonal antibody (clone 4G10) was purchased from Upstate Biotechnology. Anti-Crk monoclonal antibody was purchased from Transduction Laboratories. Anti-Crk polyclonal antisera was generously provided by Dr. Bruce J. Mayer. Anti-Crm1 polyclonal IgG was provided by Dr. Gerard Grosveld (Department of Genetics, St. Jude Children's Research Hospital, Memphis, TN). For immunofluorescence , the secondary antibody goat-anti-mouse-FITC was purchased from Jackson Immunological Inc.

Preparation of Xenopus egg extracts:

Preparation of crude interphase egg extracts has been described previously (Evans et al. 1997b). To fractionate the crude egg extract into cytosolic and membranous components, the crude extract was centrifuged further at 55 000 r.p.m. (200 000 g) in a Beckman TLS-55 rotor for the TL-100 centrifuge for 1 h. The cytosolic fraction was removed and recentrifuged for an additional 25 min. at 200 000 g. The light membrane fraction was removed and diluted into 1.5 mL of ELB and subsequently pelleted through a 5M sucrose cushion at 20 000 r.p.m. for 20 min. The heavy membrane fraction (enriched in mitochondria) was removed and the mitochondrial fraction was purified further by centrifugation of the heavy membrane through a percoll gradient consisting of 42, 37, 30 and 25% percoll in mitochondria isolation buffer (1 M sucrose, 100 mM ADP, 2.5 M KCl, 1 M DTT, 1M succinate, 1 M HEPES-KOH pH 7.5, 0.5 M EGTA, 1.5 M

mannitol) for 25 min at 25 000 r.p.m. with no brake in the TLS-55 rotor. The isolated heavy membrane fraction containing mitochondria was diluted 1:10 into ELB containing an ATP-regenerating cocktail (20 mM phosphocreatine, 2 mM ATP and 5 μg/ml creatine phosphokinase).

Reconstituted extracts were then generated from these extract fractions with purified mitochondria added to cytosol at ratios ranging from 1:20 to 1:40 (mitiochondria:cytosol). To extracts, in which nuclei were formed, light membrane was added to the cytosol/mitochondria mix at a ratio of 1:10. Nuclei were formed in these extracts by addition of demembranated sperm chromatin (~2000 nuclei/μl). These reconstituted extracts were supplemented with an energy regenerating system consisting of 2 mM ATP, 5 μg/ml creatine kinase, and 20 mM phosphocreatine (final concentrations). Recombinant proteins added to these extracts were diluted into XB buffer (protein concentrating buffer; 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM KOH–HEPES pH 7.7 and 50 mM sucrose) at the indicated protein concentrations.

DEVDase Assays

To assay caspase activity, 3 μl aliquots of each extract sample (at various time points) were incubated with 90 μl of DEVDase buffer ((50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric peptide substrate Ac-DEVD-pNA (200mM, final concentration; BIOMOL Research Labs, Inc.). Enzyme reactions were incubated at 37°C for 30. The absorbance of the

colorimetric product was measured at 405 nm using a LabSystems MultiSkan MS mictrotiter plate reader.

CSH3 binding protein pulldown assays:

The GST, Crk SH3(C), Crk SH3(C)-NES-deficient, Crk SH3(N), or B1CRS recombinant proteins were linked to glutathione sepharose beads. For pull-down assays analyzed by Western blotting, 50 μl of beads were incubated with 300 μl of extract for 1 hour at 4°C. The beads were then pelleted and washed 3-5 times with ELB. The bound proteins were eluted in 2X SDS sample buffer and separated on 7.5% SDS-PAGE gel.

Cell culture and transfection

U2OS, Cos-7 and HEK 293 cells were maintained in DMEM (Cellgro; 4.5 g/L glucose,with L-glutamine) supplemented with 10% fetal calf serum (Sigma). NIH 3T3 cells were maintained in DMEM (Cellgro; 4.5 g/L glucose,with L-glutamine) with 10% bovine calf serum. U2OS, Cos-7 and 293 were transfected using FuGene 6 (Roche) transfection reagent (4 μl/ 3T3 cells were transfected using LipofectAMINE (GIBCO-BRL; 20 μl/ 35 mm dish, 100 μl/ 100 mm dish) following the manufacturer's protocol.

Tissue culture cell apoptosis assays

Apoptosis induced by overexpression of Crk constructs was monitored using a luciferase assay as described previously (Miura and Yuan 2000). Modifications to this procedure were as follows:

293 or Cos-7 cells were platted (1.5-2.5 x 10^5 cells/ 35 mm well) in 6-well dishes and transfected with 1.5 μg of pEBB, pEBB-wt Crk, pEBB-NES(-) Crk, or pCNDA 3.0 caspase-8. Along with each gene of interest, the constitutively active luciferase reporter construct PGL3 (Promega) was cotransfected (DNA amount, 1:10). After 24-48 hours of transfection, cells were collected and washed twice with PBS. Cells were lysed in the 6-well dishes using 250 μl of lysis buffer (1% Triton X-100, 25 mM glycyl glycine pH 7.8, 4 mM EGTA, 1 mM DTT). Lysates were clarified by centrifugation at 16 000 g for 10 min. Lysates samples (100μl) are then mixed with 275 μl of ATP solution (20 mM $MgSO_4$, 5 mM ATP, 0.7 mg/ml BSA, 25 mM glycyl glycine pH 7.8). Each sample was subsequently mixed with 100 μl of 1 mM luciferin (ICN) in 25 mM glycyl glycine. Light emission was measured using a Berthold Lumat luminometer Model # LB 9501. Relative ratio of cell death was calculated as the inverse of relative luciferase activity (arbitrary units).

Apoptotic morphologies were confirmed by plating cells on coverslips and transfecting with wt and NES(-) Crk constructs. DNA was stained with Hoechst 33258 dye and characteristic apoptotic nuclear morphologies were scored. Additionally, relative rates of apoptosis were determined as follows:

pEBB wt and NES(-) Crk constructs were individually cotransfected into 293, NIH 3T3, Cos-7 cells (seeded on coverslips) with a GFP reporter construct (pEGFP N2; Clonetech; DNA amount 1:10). Coverslips were collected 24 hours post-transfection and washed 2X in phosphate-buffered saline (PBS). Cells were then fixed using 4% paraformaldehyde in PBS for 10 min. Relative rates of apoptotic killing were calculated by scoring the percent of surviving GFP cells per total number of cells in a field. For each transfectant, six fields (20 X air objective) containing at least two hundred cells were analyzed.

Immunoprecipitation from mammalian tissue culture cells:

Immune resins were generated by incubating by incubating 5 μg anti-myc or anti-FLAG (negative control) monoclonal antibodies with 30 μl of ProteinG-Sepharose (Oncogene); or 20 μg of anti-Crk antisera or pre-immune sera (negative control) with 30 μl of Protein A-Sepharose (Amersham). Antibodies were incubated with Protein A(or G)-Sepharose for 1 hour, 4°C in 200 μl of IP Buffer supplemented with 1% BSA. Before incubation with cell lysates, the resin was pelleted by centrifugation and the supernatant was removed.

To generate cell lysates, 100 mm tissue culture dishes were seeded with NIH 3T3, Cos-7, or 293 cells which were grown to 50-70% confluency. 16-20 hours post-transfection, media was removed from cells, and the cells were washed 2X with PBS. Cells were lysed in 500μl IP buffer (50 mM HEPES, pH 7.4; 1 mM EDTA; 1% NP-40; 150 mM NaCl; and 1 mM dithiothreitol) supplemented with 0.2 mM sodium

orthovanadate and aprotinin/leupeptin (5 µg/mL, final concentration). Cell lysates were sonicated by six rapid bursts from a Branson Sonifier 150 on output setting 4. and the insoluble material was pelleted by centrifugation at 16 000 g for 10 min. at 4°C. Protein concentrations of lysates were determined using the Bio-Rad protein assay dye such that they could be normalized by dilution in IP Buffer.

Cell lysates (~500 µg of total protein per sample) were incubated with immune resins for 1 hour at 4°C with gentle agitation. The resins with bound proteins were pelleted by brief centrifugation and the lysates were removed by aspiration. Subsequently, immune resins were washed four times in IP buffer; bound proteins were eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE (one-half of the total immunoprecipitated material was loaded on the gel).

Immunoblotting

Immunoblotting was performed following SDS-PAGE and transfer to PVDF membranes (Millipore). Blots were incubated with appropriate primary antisera described above and subsequently with (secondary antibodies) horseradish peroxidase-linked protein A (Amersham) or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.). Blots were developed using an enhanced chemiluminescence kit (Renaissance, Dupont NEN).

Crk Immunostaining

U2OS were seeded on sterile glass coverslips (3.5-5.0 x 10^4 cells/coverslip) and allowed to attach overnight in DMEM (Cellgro; 4.5 g/L glucose,with L-glutamine) supplemented with 10% Fetal Bovine Serum (Sigma). Following 16-24 hours of transfection, coverslips were washed twice in 1 ml of phosphate buffered saline (PBS). Cells were fixed in 4% paraformaldehyde in PBS for 10 min. Coverslips were subsequently washed 2X in PBS and then permeablized in 0.2% Triton X-100 in PBS for 10 min. on ice. Coverslips were rinsed again 2X in PBS and incubated in block solution (3% BSA , 0.02% Triton X-100 in PBS) overnight at 4°C. Coverslips were then incubated for 30 min. with primary antibody (anti-myc monoclonal) diluted 1:500 in Block buffer. Cells were rinsed 3X times in wash buffer (1% BSA, 0.02% Triton X-100 in PBS) for 5 min with gentle agitation. Subsequently, coverslips were incubated in secondary antibody (goat-anti-mouse-FITC) diluted into Block buffer 1:200 for 30 min. Excess antibody was removed by washing coverslips 3X for 5 min. in wash buffer. The final wash was supplemented with Hoechst 33258 (1 μg/mL) to stain nuclear DNA. Coverslips were mounted on slides using Anti-Fade mounting media (Molecular Probes). Crk staining and nuclear DNA staining was visualized using fluorescence microscopy.

RESULTS:

The C-terminal SH3 domain of Crk interacts with the nuclear export factor, Crm1

While our previous work had demonstrated that the SH2 domain of Crk could engage tyrosine phosphorylated Wee1, we wished to identify and characterize additional proteins that might participate in apoptotic signaling through binding to either of the two SH3 domains. In an effort to isolate such molecules, we performed a yeast two-hybrid screen of human fetal brain, human fetal liver, and human testis cDNA libraries using the full-length Crk II as bait. From a screen of one million prey clones, the only strongly positive Crk interactor to emerge was the human nuclear export protein, Crm1 (Fornerod et al. 1997; Ossareh-Nazari et al. 1997). This protein, a member of the importin family of nuclear transport receptors, mediates the export of its substrates from the nucleus to the cytoplasm in a Ran-GTP dependent manner. As Crm1 binds to a well-characterized, albeit loose, consensus sequence (Bogerd et al. 1996), we scanned the primary amino acid sequence of Crk for a similar motif that might serve as a nuclear export sequence (NES; Fig 1A). In doing so, we identified a putative NES lying within the C-terminal SH3 domain (SH3(C); Fig 1B).

To confirm the interaction between Crm1 and the C-terminal SH3 domain of Crk, we prepared a resin composed of the GST-CrkSH3(C) linked to glutathione sepharose. Subsequently, this resin was incubated with Xenopus egg extract, pelleted, washed extensively and bound proteins were resolved by SDS-PAGE for immunoblotting with anti-Crm1 sera. As shown in Figure 2A, precipitates formed using either the SH3(C) resin or a control NES resin (derived from the NES of Cyclin B1) contained Crm1 (115

15

kD), while control precipitates performed with a GST resin did not. The N-terminal SH3 domain, which lacks any observable NES-like sequence, was also unable to bind Crm1. In order to confirm that the putative NES sequence identified in Crk SH3(C) was, in fact, responsible for the observed Crm1 binding, we produced a mutant SH3(C) resin in which several residues predicted to be critical for NES function were changed to Ala (residues 263, 264, and 266). This mutant form of the SH3(C) (NES(-) SH3(C)) was unable to precipitate Crm1 from egg extracts, consistent with a role of this sequence in mediating the Crm1 interaction (Figure 2B).

To determine whether full-length Crk protein deficient in the candidate NES would be localized to nuclei more strongly than the wild type protein, we transfected myc-tagged wild type or NES(-) Crk proteins into U2OS cells and processed them for immunoflourescence with anti-myc antibodies. As shown in Fig. 3A, (with corresponding Hoechst staining of DNA shown in 3B) wild type Crk II localized throughout the cytoplasm and nucleus. In contrast, the NES (-) mutant was largely nuclear (Fig. 3C,; and corresponding Hoechst staining of DNA in 3D). These data are consistent with the identified sequence functioning as an NES and suggest that NES(-) mutant Crk entering the nucleus was retained there due to an inability to bind Crm1.

Crk II is well conserved among vertebrates with ~80% or greater similarity among mammal, frog, and chicken homologues (Evans et al. 1997b). As shown in Figure 1A, the residues within the Crk SH3(C) that constitute the NES are virtually identical in all sequenced vertebrate Crk proteins and in the human Crk-like protein CrkL. Interestingly, despite some sequence divergence, hydrophobic residues with similar

spacing in the C-terminal SH3 domain of Drosophila Crk might also be expected to confer binding to Crm1. However, this NES does not seem to be conserved in the Crk-like protein from C. elegans, CED-2, suggesting that this region of Crk may not function similarly in that organism.

A Crk-Wee1 interaction in mammalian cells is enhanced by mutation of the Crk NES

As described above, experiments in Xenopus egg extracts demonstrated that apoptotic induction by Crk depended upon binding to Wee1, a known nuclear constituent (Smith et al. 2000). Previous studies of Crk SH2 interactors in mammalian cells had identified several Crk binding-proteins, including an unidentified tyrosine phosphorylated protein of ~110 kD (for review, Matsuda and Kurata 1996). As the Wee1 (107kD) is similar in size to this band, we speculated that mammalian Crk, like its Xenopus counterpart, might engage the tyrosine kinase Wee1 within nuclei. Moreover, we predicted that any NES-deficient Crk (NES(-) Crk) entering the nucleus should be trapped there where it might interact with Wee1 to a greater degree than the wild type protein. To assess this, we transfected myc-tagged wild type or NES(-) Crk into NIH 3T3 cells and then, 24 hr later, immunoprecipitated Crk with anti-myc antibody, resolved precipitates by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. While both wild type and NES(-) Crk proteins were immunoprecipitated at similar levels (see lower panel, Fig 4) and both bound to a tyrosine phosphorylated protein of 107 kD (Fig 4 upper panel), the interaction with the NES(-) Crk was strikingly enhanced relative

to the wild type. Given the poor quality of all of the commercially available anti-Wee1 antibodies we tested, we elected to examine the possibility of a Wee1-Crk interaction directly by co-transfecting untagged wild type or NES(-) Crk with myc-tagged human Wee1. As shown in Fig 5, Wee1 co-precipitated with both Crk proteins, but again, the association with the NES(-) Crk was considerably more striking. Not surprisingly, tyrosine phosphorylation of Wee1 is required for interaction with the SH2 domain of Crk (Smith et al. 2000). Interestingly, the known site of Xenopus Wee1 phosphorylation that conforms to a Crk binding consensus, appears to be conserved in human Wee1 (Fig 5B). Indeed, treatment of cells with sodium orthovanadate to inhibit tyrosine phosphatase activity allowed detection of endogenous Crk-Wee1 interactions (Fig. 5C). Collectively, these data not only demonstrate, for the first time, an association between mammalian Crk and Wee1 proteins, but also highlight the idea that Crk restricted to the nucleus might engage a distinct signaling pathway.

NES-deficient Crk protein induces cell death

Since Crk binding to Wee1 was required for apoptosis in Xenopus egg extracts, we wished to determine whether the NES(-) and wild type Crk proteins, that differed in their ability to bind Wee1, would also display differential apoptotic activities in intact mammalian cells. Parrizas et al. reported that overexpression of wt CrkII in 293 cells caused increased death in this cell line (Parrizas et al. 1997). We found that overexpression of NES(-) Crk consistently caused a 3 to 7 fold increase in apoptosis compared to wt Crk (Fig 6). These relative death rates were measured by the luciferase

assays shown in Fig. 6 and also by scoring DNA fragmentation after Hoechst staining and by counting GFP positive surviving cells following co-transfection of GFP and Crk-expressing plasmids (data not shown). Representative fields from a GFP co-transfecton experiment are shown in Fig. 7; cultures co-transfected with the NES-deficient Crk mutant and GFP showed markedly fewer GFP-positive cells (Fig. 7C) than those transfected with the vector plasmid (Fig. 7A) or wild type Crk (Fig. 7B). Note that the GFP positive cells remaining in the NES-deficient transfectant clearly exhibited an apoptotic morphology. Collectively, these data suggest that mutation of the Crk NES both enhances Wee1 binding and promotes apoptosis.

If Crk-Wee complexes are, indeed, pro-apoptotic, we might predict that apoptotic stimuli would enhance complex formation. Since DNA damaging agents, such as ionizing radiation can either stop the cell cycle or induce apoptosis, depending on the extent of damage, we reasoned that Wee1, participating in the regulation of both cell cycle progression and cell death might be ideally positioned to transduce a radiation-responsive DNA damage signal. Accordingly, we transfected cells with myc-tagged Wee1 and subjected them to a strong damaging signal (20 Gy ionizing radiation) and then analyzed anti-myc immunoprecipitates for the presence of endogneous Crk protein. As shown in Fig. 8, X-irradiation consistently produced a modest (~2-fold) increase in Wee1-Crk complex formation, consistent with its possible involvement in signaling from DNA damage.

Localization of Crk to the cell periphery is dependent upon growth factors and Crm1-mediated nuclear export

Studies on Crk subcellular localization have placed it both in the nucleus and at the cell periphery, where it can be incorporated into either focal adhesions or in membrane ruffles at the leading edge of migrating cells. In a number of different cell lines (293, NIH 3T3, etc.), we have seen a substantial nuclear pool of wild type Crk, both by immunofluorescence and by expression of GFP-Crk fusion proteins (Figs. 3 and 9, and data not shown). If the nuclear pool of Crk is pro-apoptotic, why do healthy, normal cells exhibit nuclear Crk staining? Given reports that Crk promotes cell survival either as part of focal adhesion complexes or by Cas/Crk coupling at the edge of migratory cells, we suspect that any pro-apoptotic signals emanating from the nuclear pool of Crk are antagonized by pro-survival signals from Crk-containing structures at the cell periphery. When the NES- Crk protein was overexpressed as above, it is likely that the balance was shifted to favor cell death over cell survival. Cheresh and colleagues have observed that Crk is present at the cell periphery under normal growth conditions, in rich media (when survival signals would be expected to predominate) and leaves the periphery under starvation conditions that would, if continued, promote apoptosis. Interestingly, upon re-stimulation with insulin, Crk re-appears at the cell periphery (Klemke et al. 1998). In similar experiments, we performed immunofluorescence with anti-Crk antibodies on cells subjected to a regimen of growth factor starvation and re-stimulation. However, in order to see if Crk re-localization to the cell periphery might be

dependent upon Crk nuclear export, we performed the experiments in the presence and absence of the nuclear export inhibitor, leptomycin B.

In NIH 3T3 cells starved of growth factors, Crk protein was clearly absent from the cell periphery, but could be seen both in the nucleus and in a cytoplasmic network surrounding the nucleus (Fig.9A). Given the basal nuclear pool of Crk in these cells, it was difficult to judge whether Leptomycin B treatment alone significantly affected the amount of total Crk in the nucleus. However, the intensity of perinuclear/cytoplasmic Crk staining was noticeably reduced by leptomycin B (Fig 9B). Upon re-stimulation of the serum-starved cells with insulin or PDGF, a detectable fraction of Crk moved to sites of membrane ruffling at the cell periphery, as has been described previously (Fig. 9C and Klemke et al 1998). These data are consistent with the notion that Crk appears at the cell periphery when the cellular environment favors survival, but is absent from the periphery when the balance is tipped towards cell death. Importantly, the re-appearance of Crk at the cell periphery was entirely blocked by leptomycin B (Fig. 9D), suggesting that the peripheral Crk appearing after growth factor treatment may well have derived from the nuclear pool of Crk. Moreover, as reported by Cheresh and colleagues, Crk at the cell periphery is required for the nuclear membrane ruffling seen after growth factor stimulation. Consistent with this, we found that leptomycin B treatment, which stopped Crk from moving to the cell periphery, markedly inhibited the appearance of membrane ruffles.

The NES-deficient form of Crk requires nuclei for its acceleration of apoptosis

In Xenopus egg extracts, addition of recombinant wild type Crk produces only a mild acceleration of apoptosis relative to GST control protein (Evans et al 1997b and Fig. 9A). However, a small, but signficant and reproducible acceleration of apoptosis was seen upon addition of the NES-deficient mutant to egg extracts (Fig. 10A). If it is the nuclear population of Crk that is pro-apoptotic, the relatively increased potency of the NES(-) mutant in triggering apoptosis should depend upon the presence of nuclei. To address this issue, we exploited the ability of the Xenopus egg extract to undergo caspase activation in both the presence and absence of added nuclei. In these extracts, membrane vesicles bind to added chromatin to form enclosed nuclear structures containing nuclear pores that are fully capable of transporting macromolecules. In extracts not supplemented with chromatin and light membranes (which contribute to nuclear formation), cytoplasmic and nuclear compartments are intermixed. However, when sufficient amounts of nuclear-forming chromatin are added to these extracts, nuclear trafficking factors present in the extract allow specific nuclear accumulation of true nuclear constituents, while excluding cytoplasmic components (Walter et al. 1998). Although extracts supplemented with light membranes to form nuclei activate caspases more slowly than extracts lacking these membranes (most likely due to the presence of membrane-bound anti-apoptotic bcl-2 family members), this property of the extract did not present a problem since we could simply compare the apoptosis-inducing activities of wild type and NES(-) Crk to determine whether the *relatively* more potent activity of the mutant was evident only after nuclear compartmentalization. Accordingly, we measured caspase activation in egg extracts

supplemented with recombinant Crk (wild type or mutant) in the presence and absence of nuclei. In the absence of nuclei, wild type and mutant Crk proteins induced caspase activation (measured by cleavage of the caspase substrate DEVD-pNA) with nearly identical kinetics (Fig 10B). In contrast, the NES(-) Crk was a considerably more effective activator of caspases than the wild type protein in the presence of nuclei (Fig 10C). These data strongly support the notion that it is a population of Crk protein within the nuclear environment that promotes cell death.

Discussion

A cell's decision to live or die is influenced by diverse cellular signaling pathways. In this report we have presented data implicating nuclear Crk protein in the decision to apoptose. Having previously demonstrated a role for Crk/Wee1 complexes in promoting apoptosis in Xenopus egg extracts, we have now shown that mammalian Crk behaves similarly to Xenopus Crk in binding the tyrosine kinase Wee1 (Smith et al. 2000). Moreover, we find that in both egg extracts and mammalian cells, mutation of a binding site for the nuclear export factor Crm1 enhances Wee1 binding and renders Crk pro-apoptotic.

The C-terminal SH3 domain of Crk contains a Crm1-binding NES

Using the N-terminal SH3 domain from Crk as an affinity chromatography resin, as a bait in two-hybrid screens or as a ligand for far-western blots, a number of SH3-binding factors have been isolated and characterized (reviewed in Matsuda and Kurata 1996; Feller et al. 1998). However, similar techniques applied by a number of laboratories to the C-terminal SH3 domain have failed to yield any convincing SH3 ligands. The data presented here suggest that the C-terminal SH3 domain may not behave as a conventional SH3 domain in binding proline-rich substrates. Rather, we hypothesize that the second SH3 domain arose from gene duplication of a "real" SH3 domain, but no longer retains SH3 function. Rather, it serves as a binding site for the nuclear export factor, Crm1. This binding, observed both by two-hybrid screen and affinity chromatography, was abrogated by mutation of residues conserved among Crm1-

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binding motifs. Furthermore, introduction of this mutation into the full-length Crk protein rendered Crk mostly nuclear, consistent with this sequence functioning as an NES.

Nuclear Crk favors apoptosis

Aside from differences in subcellular localization, the wild type Crk and its NES(-) counterpart, differed in several respects: the NES mutant was considerably more efficient in binding to the tyrosine kinase Wee1 (despite equivalent cellular expression and equivalent efficiency of immunoprecipitation) and, in both Xenopus egg extracts, and mammalian cells, the NES mutant was more potent in the induction of apoptosis. Although Parrizas et al. noted enhanced apoptosis in 293 cells transfected with wild-type Crk, we found that this effect was considerably more pronounced when the NES(-) mutant was transfected. Interestingly, in Xenopus egg extracts, the relatively enhanced ability of the NES(-) mutant to induce caspase activation was dependent upon the presence of nuclei. These data strongly implicate the nuclear pool of Crk in the promotion of apoptosis.

Endogenous Crk protein has been observed in nuclei, in the cytoplasm and at sites on the cell periphery (focal adhesions and membrane ruffles; Harte et al. 1996; Hasegawa et al. 1996; Kiyokawa et al. 1998; Klemke et al. 1998; Cheresh et al. 1999; Cho and Klemke 2000). Indeed, in every cell type we examined, endogenous Crk was both cytoplasmic and nuclear, consistent with the staining we observed using exogenously expressed myc-tagged Crk (Fig. 3 and data not shown). Several reports have implicated Crk at the cell periphery (either in focal adhesions or membrane ruffles)

in promoting cell survival. For example, Cho and Klemke demonstrated that Crk-Dock180 complexes present at the cell periphery facilitated both suppression of apoptosis and actin re-organization of cells invading an extracellular matrix (Cho and Klemke 2000). More recently, Stam et al have reported that the viral form of Crk, forcibly localized to the plasma membrane by virtue of its Gag domain, enhances cell survival through activation of AKT pathways (Stam et al. 2001). In addition, cells which lose contact with the substratum or neighboring cells are known to undergo anoikis, a type of apoptotic cell death. As Crk is known to participate in focal adhesion signaling, this type of cell death, may reflect, in part, loss of peripheral Crk-mediated survival signals (Frisch and Francis 1994; Frisch et al. 1996; Harte et al. 1996; Hasegawa et al. 1996; Kiyokawa et al. 1998).

The propensity of a cell to survive or die is set by the balance between pro-apoptotic and pro-survival signals. Bearing this in mind, we propose that Crk at the periphery acts to promote cell survival, while nuclear Crk promotes apoptosis. Consistent with this hypothesis, Cheresh and colleagues have observed that Crk is present at the cell periphery under normal growth conditions, in rich media (when survival signals would be expected to predominate) and leaves the periphery under starvation conditions that would, if continued, promote apoptosis. Interestingly, upon re-stimulation with insulin, Crk re-appears at the cell periphery (Klemke et al. 1998). In similar experiments reproducing this regimen, we found that this reappearance of Crk at the cell periphery was blocked by the Crm1 inhibitor, leptomycin B . These results are

consistent with regulated Crk shuttling in response to growth factor stimulation, a possibility that merits further future investigation.

The Wee1-Crk complex and promotion of apoptosis

Given the paradigm for signaling involving adaptor proteins, it is plausible that Wee1 serves either to localize Crk to other signaling molecules in a particular subnuclear locale or to allow Crk to encounter other Wee1-associated proteins. Although a proportion of the wild type protein can be seen within nuclei, we did not observe high levels of Crk-Wee1 complexes after wild type protein transfection. While the enhanced binding of the NES(-) mutant to Wee1 almost certainly reflects a greater nuclear accumulation of this protein, it is also possible that Crm1 binding to the C-terminal SH3 domain in the wild type protein in some way impedes binding of the SH2 domain to Wee1. Alternatively, Crm1 binding may bring Crk protein to a intra-nuclear localization (e.g. proximal to the nuclear pores) inaccessible to Wee1. Careful examination of the intranuclear distribution of the wild type and mutant Crk proteins coupled with future experiments to determine if Wee1 and Crm1 can bind Crk simultaneously should help to answer these questions.

Given the fact that Crk can only bind tyrosine phosphorylated Wee1, we would speculate that apoptotic stimuli might stimulate Wee1 tyrosine phosphorylation, thereby promoting Wee1/Crk complex formation and eventual cell death. While it is clear that all apoptotic stimuli will not trigger Wee1-Crk complex formation, we should be able to detect enhanced complex formation in response to particular apoptotic triggers. Indeed, complex formation between Wee1 and Crk was enhanced following treatment of cells

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with ionizing radiation. This raises the possibility that radiation (and probably other apoptotic stimuli) either stimulates a Wee1-directed tyrosine kinase or promotes Wee1 autophosphorylation. In this regard, it is interesting to note that mutation of Xenopus Wee1 at tyrosines 90, 103, and 110 abrogated Crk binding (Smith et al. 2000). As Wee1 can auto-phosphorylate at all three of these sites in vitro, it may be that apoptotic signaling molecules can stimulate Wee1 autophosphorylation. Although the nature of such a stimulatory molecule(s) is currently unclear, it is interesting to note that the Ser/Thr kinase Mos can promote Wee1 autophosphorylation in the context of the first embryonic cell cycle; this raises the possibility that a distinct Ser/Thr kinase, activated under apoptotic conditions, might also act to stimulate Wee1 autophosphorylation (Murakami et al. 1999). Alternatively, apoptotically-regulated Wee1-binding proteins might control its propensity to autophosphorylate. Finally, although Wee1 can autophosphorylate at sites required for Crk binding, it is certainly possible that another kinase phosphorylates those sites on Wee1 in vivo.

If Crk at the cell periphery does favor cell survival, increasing the nuclear pool of Crk could trigger cell death simply by titrating N-terminal SH3-binding factors necessary to transmit survival signals away from the cell periphery, sequestering them within nuclei. Under these circumstances, Wee1 might act simply to anchor the nuclear Crk, aiding in sequestration. However, in Xenopus egg extracts (which clearly lack a "periphery" of any sort), immunodepletion of either Crk or Wee1 actually prevents apoptosis, suggesting that a pro-apoptotic pathway employing these proteins operates in these extracts (Smith et al. 2000). While further experimentation will be necessary to

distinguish between these mechanisms of Crk/Wee1 action, it will be interesting to compare the population of proteins bound to the N-terminal SH3 domain of Crk in nuclei to those bound to Crk located at the cell periphery. If a distinct pro-apoptotic Crk/Wee1 signaling pathway exists, we might expect to see different SH3 binders engaged in these two compartments. Indeed, one attractive candidate for transmission of a pro-apoptotic signal is the nuclear tyrosine kinase, c-abl, a known binder of the N-terminal SH3 domain of Crk that can both impinge on apoptotic signaling and shuttle to and from nucleus (Ren et al. 1994; Taagepera et al. 1998; Gong et al. 1999). Indeed, when the cytoplasmic oncogenic variant of abl, Bcr-Abl, is forcibly localized to the nucleus, apoptosis ensues (Vigneri and Wang 2001). Future experiments will be aimed at examining this and other SH3 interactors to elucidate the pathway by which nuclear Crk contributes to cell death.

Acknowledgements

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Figure Legends

Fig. 1 Crk II contains a putative nuclear export sequence (NES) in its SH3(C) domain. The Crm1-binding consensus sequence, or NES, is defined by Bogerd et al. (for the human T-cell leukemia virus type 1 protein Rex) as **L--X$_{2-3}$--{F,I,L,V,M}--X$_{2-3}$--L--X--L** (1996). This definition has been expanded to include substitutions of valine or isoleucine for leucine (e.g. Yang et al. 1998). **(A)** Displayed here is an alignment of the putative nuclear export sequences from an array of metazoan Crk homologues: human Crk II (huCrk), avian Crk II (avCrk), Xenopus laevis Crk II (xenlaCrk), human Crk-like protein (huCRKL), Drosophila melanogaster Crk II (dmCrk), and C. elegans Crk II (CED-2). **(B)** A map of the Crk Src homology (SH) domains within its primary amino acid sequence: SH2 domain (residues 13-118), N-terminal SH3 domain (SH3(N); residues 132-192), and C-terminal SH3 domain (SH3(C); residues 256-296). Note the putative NES is highlighted within the SH3(C) domain.

Fig. 2 Crm1 binds the C-terminal SH3 of Crk. **(A)** Equivalent levels of GST fusions of Crk SH3(N), Crk SH3(C), Cyclin B1 NES (B1NES), and GST alone were bound to glutathione-sepharose resin. Resins were incubated with egg extracts in order to precipitate specific binding proteins. Non-specifically associated material was removed by washing in ELB three times. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and detected by immunoblotting using a polyclonal anti-Crm1 anti-sera. Positive controls for Crm1-binding include Cyclin B1 NES resin and egg extract cytosol (40 μg). Negative controls for Crm1-binding include Crk SH3(N) and GST. **(B)** This

same protocol was performed using a GST fusion of the mutated Crk SH3(C) domain (NES(-) SH3(C)) and similar controls as listed above.

Fig. 3 U20S cells, seeded on glass coverslips, were transiently transfected with 1.0 µg of either (A,B) pEBB myc-wt Crk or (C,D) pEBB myc-NES(-) Crk. Coverslips were processed for immuno-staining with a anti-myc monoclonal antibody followed by secondary staining with a goat-anti-mouse-FITC secondary antibody as described in *Materials and Methods*. (A) and (C) display transfected Crk immuno-staining, while (B) and (D) show Hoechst 33258 staining of nuclear DNA corresponding to A and C respectively.

Fig. 4. A tyrosine-phosphorylated band of ~107 kD co-precipitates with NES(-) Crk. NIH 3T3 cells were transfected with pEBB myc-wt or myc-NES(-) Crk. Cells were lysed and lysates (~500 µg of total protein per sample) were incubated with immuno-resins consisting of anti-myc or control (anti-FLAG) monoclonal antibodies bound to Protein G-sepharose. Immunoprecipitated material was washed 3 times in IP buffer to remove non-specifically associated material. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting using an anti-phosphotyrosine antibody (upper panel) or anti-Crk antisera (lower panel; demonstrates similar amounts of wt and NES(-) Crk in immunoprecipitates).

Fig. 5. Wee 1 co-precipitates with NES(-) Crk. (A) untagged pEBB wt Crk or NES(-) Crk were cotransfected with myc-Wee1 in NIH 3T3 cells. Cells were lysed and lysates

(~500 μg of total protein per sample) were incubated with immuno-resins consisting of anti-Crk antisera or pre-immune sera bound to Protein A-sepharose. Non-specifically bound material was removed by washing resins three times with IP buffer. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting with an anti-myc antibody (*upper panel*) or an anti-Crk antibody (*lower panel*; demonstrates similar amounts of wt and NES(-) Crk in immunoprecipitates). **(B)** An alignment of the putative Crk SH2-binding consensus sequence (YXXP/L) in Xenopus (XWee1; residues 398-411) and human (hu Wee1; 487-500) Wee1 homologues. **(C)** Endogenous Wee 1 coprecipitates with endogenous Crk in orthovanadate treated NIH 3T3 cells. Indicated samples were treated with 50μM sodium orthovanadate for 16 hrs. Cells were lysed and lysates (~1 mg total protein per sample) were incubated with immuno-resins consisting of anti-Crk or preimmune polyclonal antibodies bound to Protein A-sepharose. Immunoprecipitated proteins were washed three times in IP buffer to remove nonspecifically bound material. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting using an anti-phosphotyrosine antibody.

Fig. 6. Overexpression of NES(-) Crk in tissue culture cells increases apoptotic cell death. pEBB (empty vector), pEBB wt Crk or NES(-) Crk (1.5 μg DNA/35 mm well) was contransfected with constituitively active luciferase reporter construct (DNA amount 1:10). After 24 hours of transfection, cells were lysed and lysates were processed for

luciferase activity. Graph shows relative cell death plotted as the inverse of luciferase activity (arbitrary units).

Fig. 7 Cells transfected with NES(-) Crk exhibit apoptotic cell death. (**A**) pEBB (empty vector, (**B**) pEBB wt or (**C**) NES(-) Crk constructs (1 μg/well) were individually cotransfected into 293 cells (20,000 cells seeded on coverslips) with a GFP reporter construct (pEGFP N2; Clonetech; DNA amount 1:10). Cells (on coverslips) were processed according to *Materials and Methods*. *Panels on left display GFP positive cells, while panels on right demonstrate total cell population by phase contrast.*

Fig. 8 Gamma irradiation enhances Crk-Wee1 association. NIH-3T3 cells were transfected with pcDNA3 (empty vector) or myc-Wee1. After 16 hours, the indicated samples were mock treated or exposed to 20 Gy gamma rays from a Cs^{137} source. Cells were lysed and lysates (~450 μg total protein) were incubated with immuno-resins consisiting of anti-Crk or preimmune polyclonal antibodies bound to Protein A-sepharose. Immunoprecipitated proteins were washed three times in IP buffer to remove nonspecifically bound proteins. Bound proteins were eluted by boiling samples in SDS-PAGE sample buffer and detected by immunoblotting with: *Upper panel:* anti-c-myc monoclonal antibody; *Lower panel:* anti-Crk monoclonal antibody.
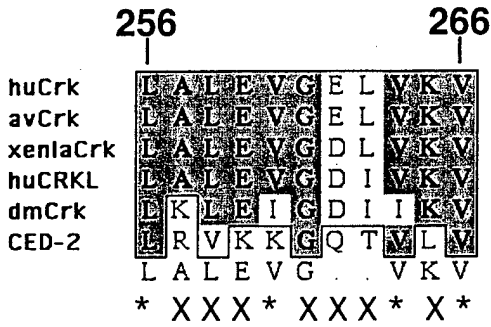
Fig. 9. Crk shuttles to the cell periphery in a Leptomycin B-sensitive manner, following growth factor stimulation. NIH 3T3 cells were seeded on coverslips and subsequently

serum starved for 24 hours (**A**). Certain cell samples (**B** and **D**) were then treated with the Crm1 inhibitor Leptomycin B (5 ng/mL) or vehicle (**A** and **C**) for four hours. Cells were then refed with (25 μg/mL) insulin (**C** and **D**) or control media (**A** and **B**) for fifteen minutes. Coverslips were subsequently processed for immunostaining with anti-Crk antisera.

Fig. 10. Addition of NES(-)Crk to egg extracts accelerates apoptotic activity. (**A**) GST, wt Crk, or NES(-) Crk were added (500 ng/μL) to crude extracts containing exogenous nuclei (by addition of demembranated sperm chromatin); alternatively, NES(-) Crk or wt Crk were added (500 ng/μL) to reconstituted extracts consisting of (**B**) purified cytosol and mitochondria or (**C**) purified cytosol, mitochondria, light membranes and sperm chromatin (to form nuclei). During a room temperature incubation, extract aliquots were taken at indicated times and processed for cleavage of the caspase substrate DEVD-pNA.
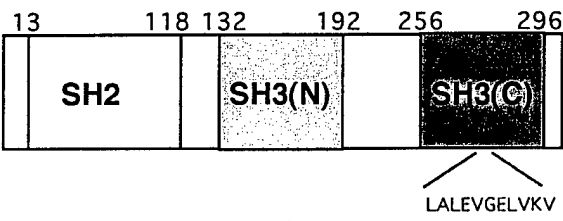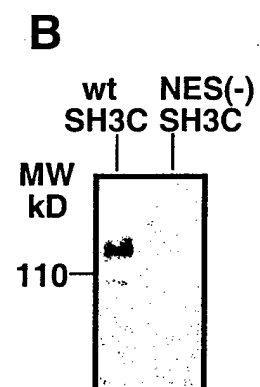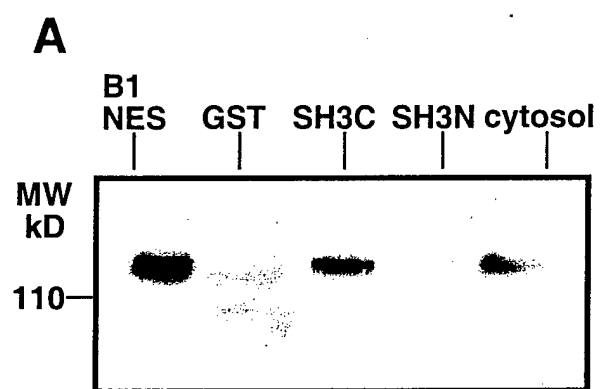
Fig 1

A

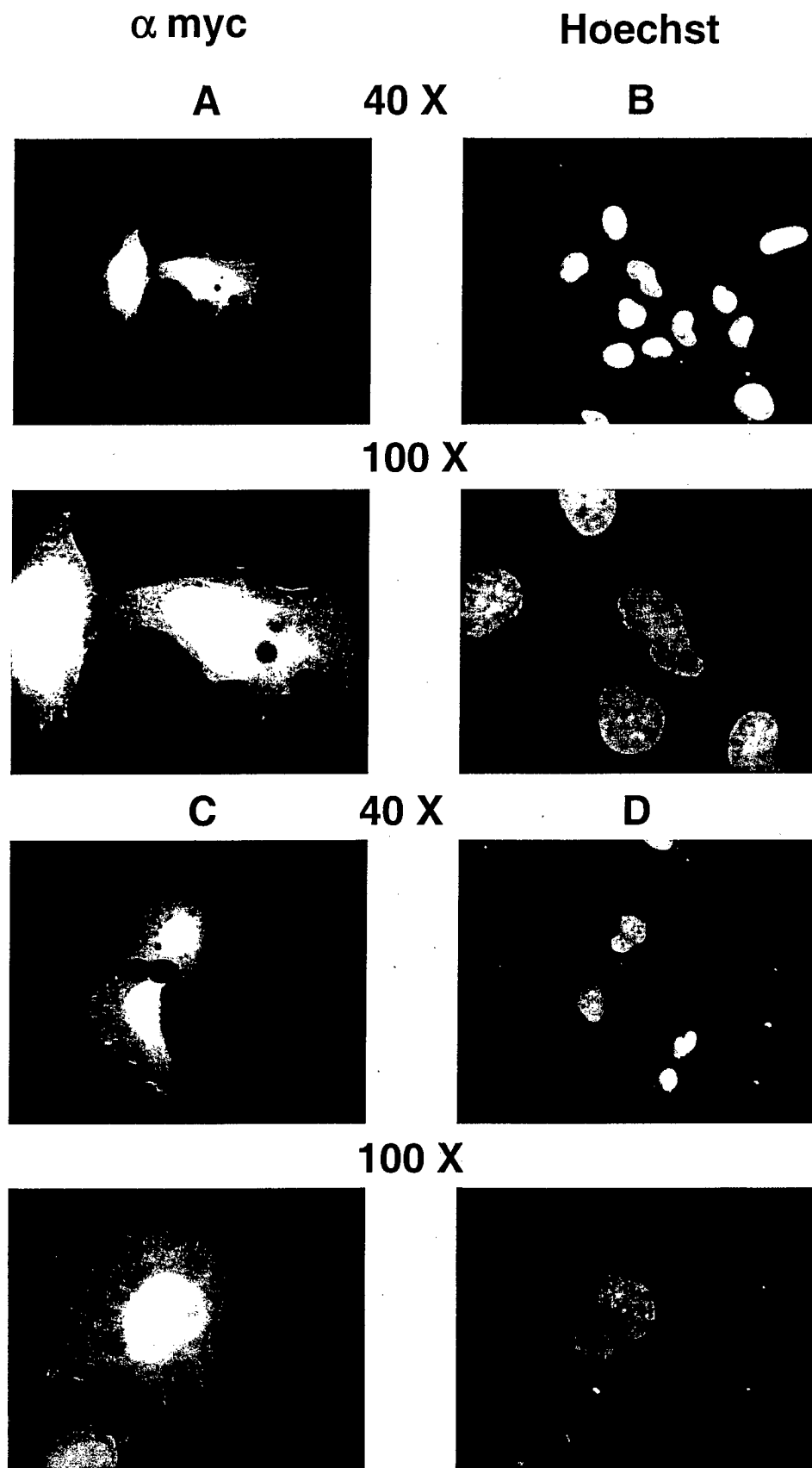256   266

huCrk   L A L E V G E L V K V
avCrk   L A L E V G E L V K V
xenlaCrk   L A L E V G D L V K V
huCRKL   L A L E V G D I V K V
dmCrk   L K L E I G D I I K V
CED-2   L R V K K G Q T V L V

           L A L E V G . . V K V

           * X X X * X X X * X *

B

13   118 132   192 256   296

| SH2 | SH3(N) | SH3(C) |

LALEVGELVKV

Fig 2.

A

MW kD

B1 NES   GST   SH3C   SH3N cytosol

110—

B

MW kD

wt   NES(-)
SH3C   SH3C

110—

Fig 3

α myc   Hoechst

A   40 X   B

100 X

C   40 X   D

100 X

Fig 4

anti-P-Tyr blot

myc cont. myc cont.

| | | |

MW   wt crk   NES⁻crk
kD

110 —

79 —

62 —

anti-Crk blot

49 —

Fig 5

anti-myc blot

A

P.I. α Crk P.I. α Crk

wt Crk   NES⁻Crk

MW
kD

110 —

79 —   ⟵

62 —

anti-crk blot

49 —

B

487   492   497

hu Wee1   VLQENYTHLPKADI
XWee1   ILQEDYSQLPKADI
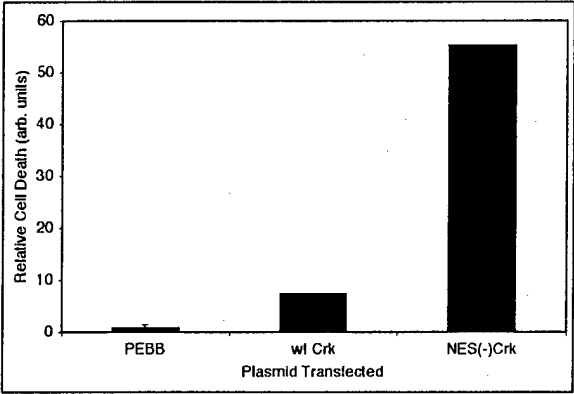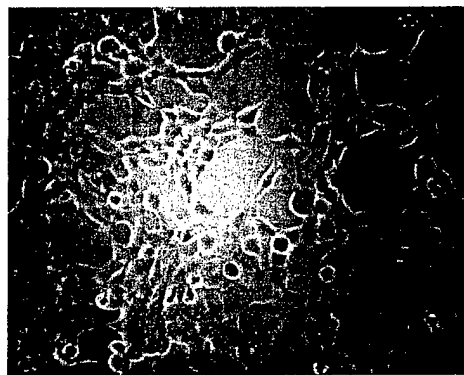
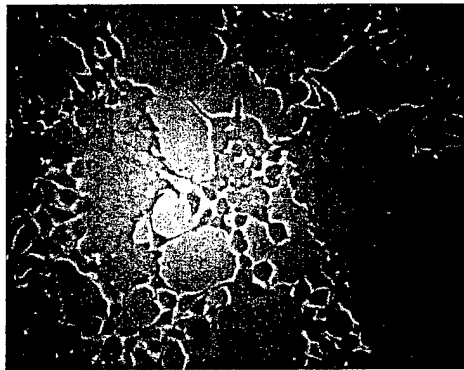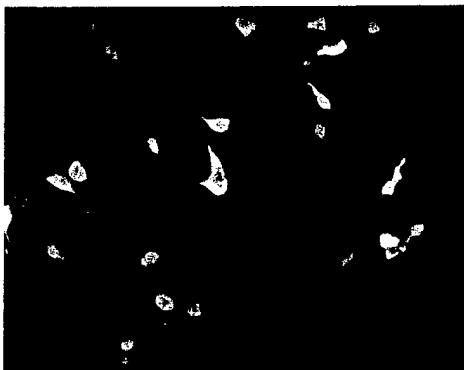   Y x x L

C

- Na₃VO₄   +Na₃VO₄

PI α Crk   PI α Crk

                    ⟵

Fig 6

Fig.7 .

GFP

Phase
Contrast

A

B

C
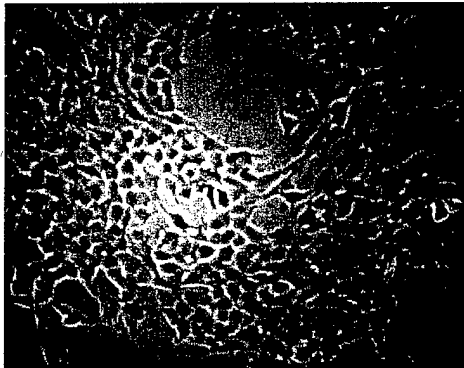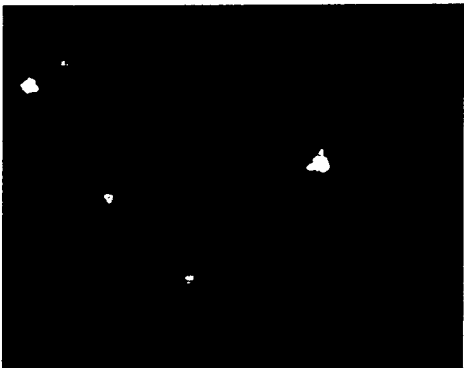
. Fig 8
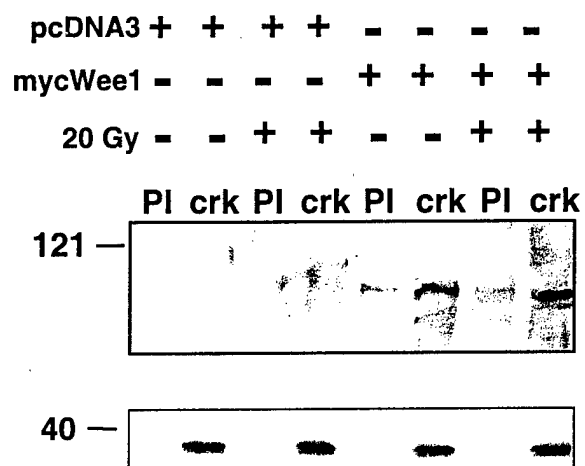
Fig 9
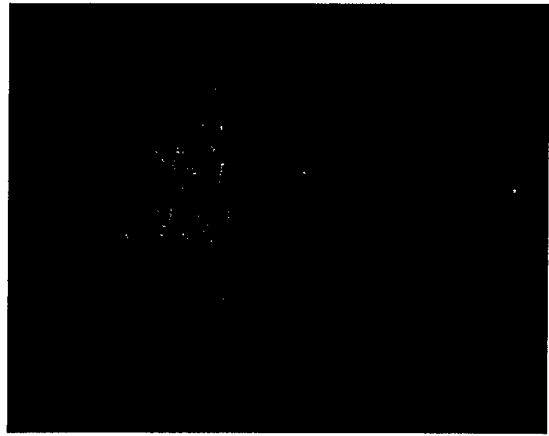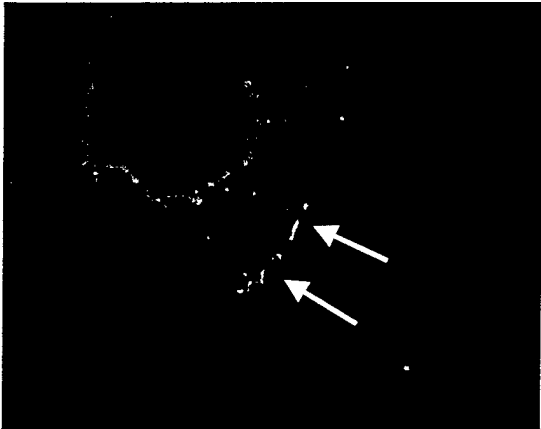
Fig 10

A

B

C

Scythe: a novel reaper-binding apoptotic regulator

Kenneth Thress, William Henzel[1], Wendy Shillinglaw[1] and Sally Kornbluth[2]

Department of Pharmacology and Cancer Biology, Duke University Medical Center, C366 LSRC, Research Drive, Durham, NC 27710 and [1]Division of Protein Chemistry, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990, USA

[2]Corresponding author
e-mail: kornb001@mc.duke.edu

Reaper is a central regulator of apoptosis in *Drosophila melanogaster*. With no obvious catalytic activity or homology to other known apoptotic regulators, reaper's mechanism of action has been obscure. We recently reported that recombinant *Drosophila* reaper protein induced rapid mitochondrial cytochrome *c* release, caspase activation and apoptotic nuclear fragmentation in extracts of *Xenopus* eggs. We now report the purification of a 150 kDa reaper-interacting protein from *Xenopus* egg extracts, which we have named Scythe. Scythe is highly conserved among vertebrates and contains a ubiquitin-like domain near its N-terminus. Immunodepletion of Scythe from extracts completely prevented reaper-induced apoptosis w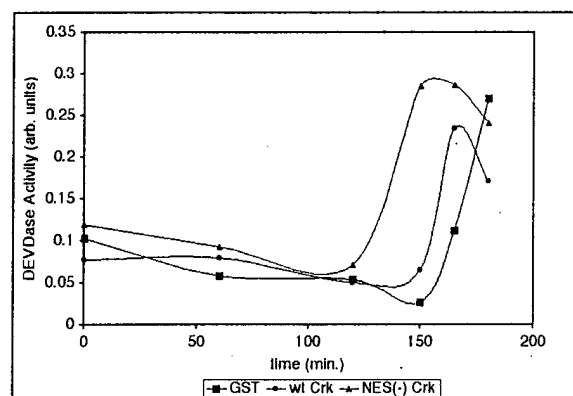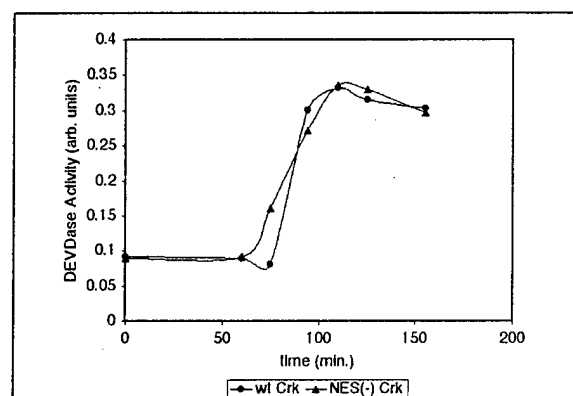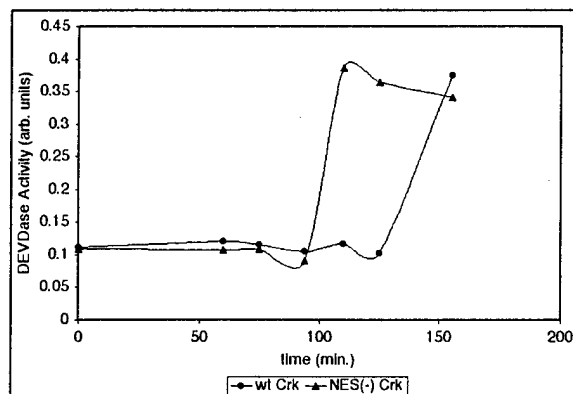ithout affecting apoptosis triggered by activated caspases. Moreover, a truncated variant of Scythe lacking the N-terminal domain induced apoptosis even in the absence of reaper. These data suggest that Scythe is a novel apoptotic regulator that is an essential component in the pathway of reaper-induced apoptosis.
Keywords: apoptosis/reaper/Scythe/*Xenopus*

Introduction

Apoptosis is a form of cell death which eliminates superfluous or damaged cells without disturbing overall tissue architecture. Apoptotic elimination of cells is a common feature of metazoan development; in many cell lineages, a significant proportion of the cells initially generated are removed by apoptosis before embryonic development is complete (Ellis *et al.*, 1991; Steller, 1995). Moreover, in the adult organism, apoptosis contributes to tissue homeostasis, immune function and prevention of a host of pathologies.

Apoptotic cell death generally involves activation of a family of proteases known as caspases (Chinnaiyan and Dixit, 1996). These aspartate-directed cysteine proteases are synthesized as inactive zymogens. Once activated, they are thought to undermine the structural integrity of the cell through cleavage of key structural protein substrates. Several additional modulators of the apoptotic process have been identified, including pro- and anti-apoptotic bcl-2 family members, and proteins interacting directly with caspases or with the zymogenic pro-caspases (e.g. FADD, TRADD, Apaf-1, IAPs: Nunez and Clarke, 1994; Reed, 1994; Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995; Deveraux *et al.*, 1997; Seshagiri and Miller, 1997; Zou *et al.*, 1997).

The signaling events which favor activation of particular apoptotic regulators are currently a focus of intense investigation. In several systems, transmission of a pro-apoptotic signal results in release of cytochrome *c* from the intermembrane space of mitochondria to the cytoplasm (Liu *et al.*, 1996; Kluck *et al.*, 1997a). Cytoplasmic cytochrome *c* then serves as a co-factor for caspase activation, leading ultimately to cell death (Zou *et al.*, 1997). Bcl-2 family members can prevent both cytochrome *c* release (Kluck *et al.*, 1997b; Yang *et al.*, 1997) and subsequent apoptosis (Hu *et al.*, 1998; Rosse *et al.*, 1998) in different contexts. Thus, the apoptotic process is vulnerable to regulation at many levels.

In a screen to identify novel apoptotic regulators in *Drosophila*, White *et al.* (1994) identified a 65 amino acid protein which they named reaper. Transcriptional induction of reaper consistently precedes the onset of programmed cell death in flies, and deletion of reaper prevents all programmed cell deaths. Furthermore, ectopic expression of reaper in lepidopteran cells promotes rapid apoptosis (Pronk *et al.*, 1996; White *et al.*, 1996). These findings established reaper as a key regulator of apoptosis in flies, and genetic data places reaper upstream of caspase activation, although the molecular intermediates between reaper and caspase activation have not been elucidated.

To date, no reaper homologs have been discovered in vertebrate species. However, we recently reported that recombinant *Drosophila* reaper protein induces rapid apoptosis upon addition to cell-free extracts prepared from *Xenopus* eggs (Evans *et al.*, 1997). Addition of reaper triggered many hallmark events of apoptosis including mitochondrial cytochrome *c* release, caspase activation, nuclear fragmentation and the characteristic DNA 'laddering' seen in apoptotic cells of diverse origin. Furthermore, at high stoichiometric ratios of bcl-2 to reaper, these processes were inhibitable (Evans *et al.*, 1997). These findings suggested that reaper-responsive pathways were conserved between arthropods and vertebrates.

Reaper-induced mitochondrial cytochrome *c* release required the presence of cytosol, suggesting that intermediary factors acted between reaper and the mitochondria. In order to identify such factors, we have purified proteins from *Xenopus* egg extracts that interact physically with reaper. We report here a reaper-interacting molecule, Scythe, which is required for both mitochondrial cytochrome *c* release and phenotypic apoptosis in response to reaper. Moreover, we show that a C-terminal fragment of Scythe can act as an independent inducer of apoptosis. Collectively, these data establish Scythe as a critical mediator of reaper-induced apoptosis.

Results

Identification of a reaper-interacting protein

Since reaper is a small protein without obvious catalytic activity, we hypothesized that it might act through direct interaction with downstream apoptotic effectors. In order to isolate such reaper interactors, we used GST–reaper protein linked to glutathione–Sepharose as a 'bait' to retrieve interacting proteins from *Xenopus* egg extracts. After incubation in egg extracts, these GST–reaper beads (or the control bait, GST beads) were pelleted and washed extensively. For preliminary identification, all proteins which remained bound to GST or to GST–reaper were modified chemically using a succinimide ester of biotin and then resolved by SDS–PAGE. After transfer to nitro-cellulose, the biotinylated proteins were visualized by staining with horseradish peroxidase (HRP)–streptavidin. As shown in Figure 1A, a prominent doublet of 148/150 kDa interacted specifically with GST–reaper.

We scaled up our purification protocol to obtain Coomassie Blue-stainable levels of the 148/150 kDa proteins for microsequencing. Starting with 700 µg of GST–reaper and 15 ml of *Xenopus* egg extract (40 mg/ml total protein), we obtained ~2–3 pmol of the reaper-binding proteins. After SDS–PAGE, proteins in these bands were subjected to tryptic and Lys-C digestion, and eluted peptides were resolved by HPLC. Mass spectrometric analysis indicated that the proteins present in the closely spaced doublet were very highly related, possibly representing closely related isoforms or post-translationally modified variants of each other. Sequencing of peptides derived from the upper band of the doublet revealed it to be highly related to a previously sequenced human open reading frame (ORF), called BAT3 (HLA-<u>B</u>-<u>a</u>ssociated <u>t</u>ranscript <u>3</u>), identified in a chromosomal walk through the HLA-B region of the MHC III locus (DDBJ/EMBL/GenBank accession No. M33519; Spies *et al.*, 1989; Banerji *et al.*, 1990). We have named the protein encoded by this transcript 'Scythe.' Overall, Scythe is not markedly homologous to any other proteins in the DDBJ/EMBL/GenBank database. However, the N-terminal 80 amino acids bear 37% identity and 54% similarity to the human ubiquitin protein.

Using a cDNA probe encoding the human Scythe protein for low stringency hybridization of a *Xenopus* library, we isolated a candidate *Xenopus* homolog of Scythe (Figure 1B). Sequences from 11 different tryptic and Lys-C peptides, derived from both the lower and upper bands of the 148/150 kDa doublet, were found to be identical to sequences encoded by the *Xenopus* Scythe clone (see Figure 1B). Overall, *Xenopus* Scythe is 57% identical and 62% similar to human Scythe.

To confirm that Scythe could indeed bind to reaper, we transcribed and translated *Xenopus* Scythe *in vitro*, added it to egg extracts, and incubated these extracts with GST beads or GST–reaper beads. The *in vitro* translated radiolabeled Scythe protein bound tightly to the GST–reaper protein, but not to GST (Figure 1C); the reaper–Scythe interaction was maintained even after washing in buffers containing 1 M NaCl (data not shown). Scythe could also bind directly to reaper in the absence of egg extract, although we observed some background binding of Scythe to GST under these conditions (Figure 1C).

Taken together, these data suggest that Scythe is a *bona fide* reaper-interacting protein.

The C-terminal 312 amino acids of Scythe can trigger apoptosis

Since reaper induces apoptosis in *Xenopus* egg extracts, we were interested in the possibility that overproduction of Scythe in these extracts might also trigger apoptosis. However, when we added baculovirus-produced full-length Scythe (final concentration, 600 ng/µl) to extracts containing nuclei, the nuclear morphology of synthetic nuclei formed around sperm chromatin templates was unaltered. In addition, exogenous Scythe did not induce detectable caspase activation (data not shown). Given these data, we reasoned that reaper binding might alter the conformation of Scythe, allowing downstream pro-apoptotic effectors to interact with normally inaccessible domains of Scythe. Consistent with this hypothesis, we found that a bacterially expressed protein consisting of GST fused to the 312 C-terminal amino acids of Scythe (ScytheC312) was a potent inducer of apoptosis; upon addition to *Xenopus* egg extracts, 600 ng/µl recombinant ScytheC312 induced apoptotic nuclear fragmentation and DEVDase activation with a time course very similar to that previously reported for reaper-induced apoptosis in these extracts (Figure 2A and B) (Evans *et al.*, 1997). The photomicrographs shown are highly representative in that apoptotic nuclear fragmentation was synchronous within a given sample, proceeding to completion within 10 min, even at concentrations of nuclei as high as 1000/µl. A titration of ScytheC312 protein added to the extract is shown in Figure 2C; note that 600 ng/µl Scythe protein is roughly equivalent to the concentration of Scythe protein found endogenously in the extract (data not shown). The specificity of the ScytheC312 effect is highlighted by the fact that further truncation of the C-terminal portion of Scythe to include only the C-terminal 235 amino acids (ScytheC235) led to a loss of apoptotic activity. Moreover, a Scythe fragment derived from the N-terminal 435 amino acids of the protein (ScytheN435) also lacked the ability to induce either morphological apoptosis or caspase activation (Figure 2A and B). Interestingly, *in vitro* translated, ^{35}S-labeled ScytheC312 protein could bind recombinant reaper, while neither ScytheN435 nor ScytheC235 retained this ability (Figure 3). Taken together, these data suggest that either addition of reaper or removal of the N-terminal 824 amino acids can activate the pro-apoptotic activity of Scythe and that the biologically active fragment of Scythe interacts physically with reaper.

Mitochondrial cytochrome c release in response to ScytheC312 requires accessory cytosolic factors

Since reaper requires cooperating cytosolic factors to trigger mitochondrial cytochrome *c* release, we hypothesized that Scythe might be a cytochrome *c*-releasing factor. Indeed, addition of ScytheC312 to crude egg extracts accelerated release of cytochrome *c* from the mitochondria relative to controls (Figure 4A). ScytheC312 was also able to trigger cytochrome *c* release when added to a mixture of isolated cytosol and mitochondria (Figure 4B). Unlike ScytheC312, Sf9-produced full-length Scythe did not induce mitochondrial cytochrome *c* release in either crude extract or isolated cytosol (Figure 4A and B); in

A

Extract: - - + +
Beads: GST RPR GST RPR

200 kD

97 kD

68 kD

C

FBS   Extract

GST GST-reaper GST GST-reaper

200 —

97 —

68 —

45 —

← Scythe

B

```
   1  MAANEKMEVT VKTLDSQTRT FTVETEISVK DFKAHISSDV GISPEKQRLI YQGRVLQEDK KLKEYNVDGK VIHLVERAPP QTQPSTGGPS TSSSTSPTSS

 101  NAAPVPGAPE RNGNSYVMVG TFNLPHVMSG LVRQQRPSVS TVNGNDGSTL DVHINLDQQL PVQSEPRVRL VLAQHILQDI QRILDRLEGQ AVNEQAAEPM

 201  DTAESEGEAS SRETLPQTTQ NTDGQSNTTP TSHPSPSEYV EVLQSLSRVE ERLAPFMQRY REILSSATSD AYENQEEREQ SQRIINLVGE SLRLLGNALV

 301  AVSDLRCNLS SASPRHLHVV RPMSHYSGPM LLQQAAIPIQ INVGTTVTAT GNGTHAGHMP SDGNAAQPPS TNTSEPQRPN TENQPPSNGE RPASDAPPTS

 401  VPHPHPRVIR ITHQTVEPVM MMHMNIQDSA SGGPTTIPPP TAGHGGSAHI HMPGLPPEFM QAISHQITQQ AMAAASGQQI PGFQAPPRFV PTRPAAPSFQ

 501  FQPGTATTPP GPGGATTTVP GATVGPAGNA SLAQMISGLV GQLLMHPVVV AQGGSSTSSS TSSSTFTSTS SSASSSSSTD TTSTTTTSST ANPTVSSVPS

 601  SQPPPGTDQH LSQLLGSLLG TASSGMSNIT MGSPSITVTV PGMPAFLQGV TDILQATQTV PVSTAPTQSA SQAPPPSSPP PPPAHSSPPP AAAPESLPPE

 701  FFTSVVQGVL SSMLGSLSAA DQSGTESIAA FIQRLSGTHN IFQPDAEGPG GFFGDLLTLI CHNFSLVDMV MLLHGHSQPL QNLQPQLRSF FLQEYLHQVD

 801  PTPNNIQMAS RNLTNGLEEY IRESFASVTV RDDVDITRTN IEFLQDQFNR ITTHILHCAD STFGQRLLEM CNQSLFEWLA LNLYCLRGDQ NALTSVINER

 901  IRRLSLDVSP VLVSWVTSVL SLRLQVLLGQ MPVTEGEIQR HVRRVGDAPQ VPEPSSQEQP METMPVDCQN GAASPVLATH SGGVLFLPPQ SSVPTICPDS

1001  DHPTQEDGGS EQWAASVPPE WVPVIRQDMQ NQRKIKQQPP LSDAYLSGMP AKRRKTMQGE GPHLSLSEAV SRAMKATGAK PESSAECVRR ELDNSEAQGR

1101  YREQLCQDIQ KTLQDNESYS AQRFPNTQRA FRGDP*
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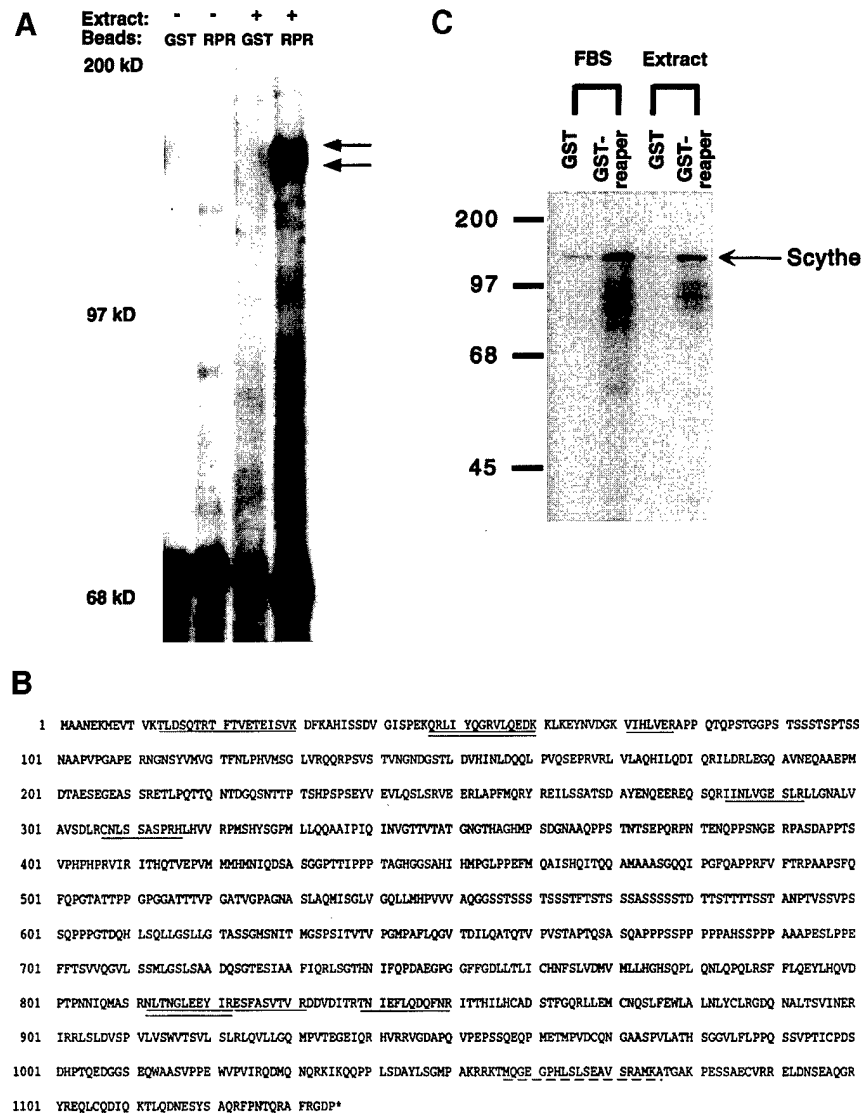
Fig. 1. (A) A protein doublet of 148/150 kDa interacts specifically with reaper (RPR) beads. Recombinant GST–reaper protein on glutathione–Sepharose beads ('RPR beads') was incubated with *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed twice with egg lysis buffer (ELB), twice with NaBicarb buffer, resuspended in NaBicarb and incubated with a succinimide ester of biotin for 1 h at room temperature. The beads were then pelleted, washed twice with ELB, resuspended in SDS sample buffer and processed for a Western blot using an HRP-linked streptavidin antibody. (B) Predicted amino acid sequence of Scythe ORF. Eleven tryptic and Lys-C peptides that were found in the two different Scythe isoforms (148 and 150 kDa) are indicated as follows: single underlining = 150 kDa; double underlining = 148 kDa; dotted underlining = 148 + 150 kDa. (C) *In vitro* transcribed/translated Scythe protein interacts with reaper. ^{35}S-Labeled Scythe protein was incubated with GST or GST–reaper beads in the presence of either heat-inactivated FBS or *Xenopus* egg extract for 30 min at room temperature. Beads were then washed three times with ELB, resolved by SDS–PAGE and processed for autoradiography.

several experiments, we observed some suppression of cytochrome *c* release by the full-length Scythe protein. In contrast to the results obtained in the presence of cytosol, ScytheC312 did not promote cytochrome *c* release from isolated mitochondria in buffer (in the absence of other cytosolic proteins), even in the presence of recombinant reaper (Figure 4C). These data suggest that other accessory cytosolic factors are required to promote cytochrome *c* release.

Scythe is required for reaper-induced apoptosis

To evaluate the role of Scythe in reaper-induced apoptosis, we wished to deplete endogenous Scythe from extracts and determine whether the depleted extracts retained the ability to induce apoptosis in response to reaper. For

immunodepletion, we produced several antisera directed against Scythe. Antisera directed against a peptide consisting of the 40 C-terminal amino acids of Scythe (anti-peptide sera) and antisera directed against ScytheC312 both recognized a 150 kDa doublet on immunoblots of *Xenopus* egg extracts (data not shown). Using the anti-ScytheC312 sera coupled to protein A–Sepharose, we performed three successive rounds of immunoprecipitation of Scythe from aliquots of egg extract. As shown in Figure 5A, these extracts were fully depleted of Scythe, as indicated by Western blotting with the anti-peptide antisera (this gel was not of sufficient resolution to separate the doublet). Similar depletions with pre-immune sera did not detectably remove any Scythe protein from the extract. Depletion of Scythe prevented reaper-induced DEVDase
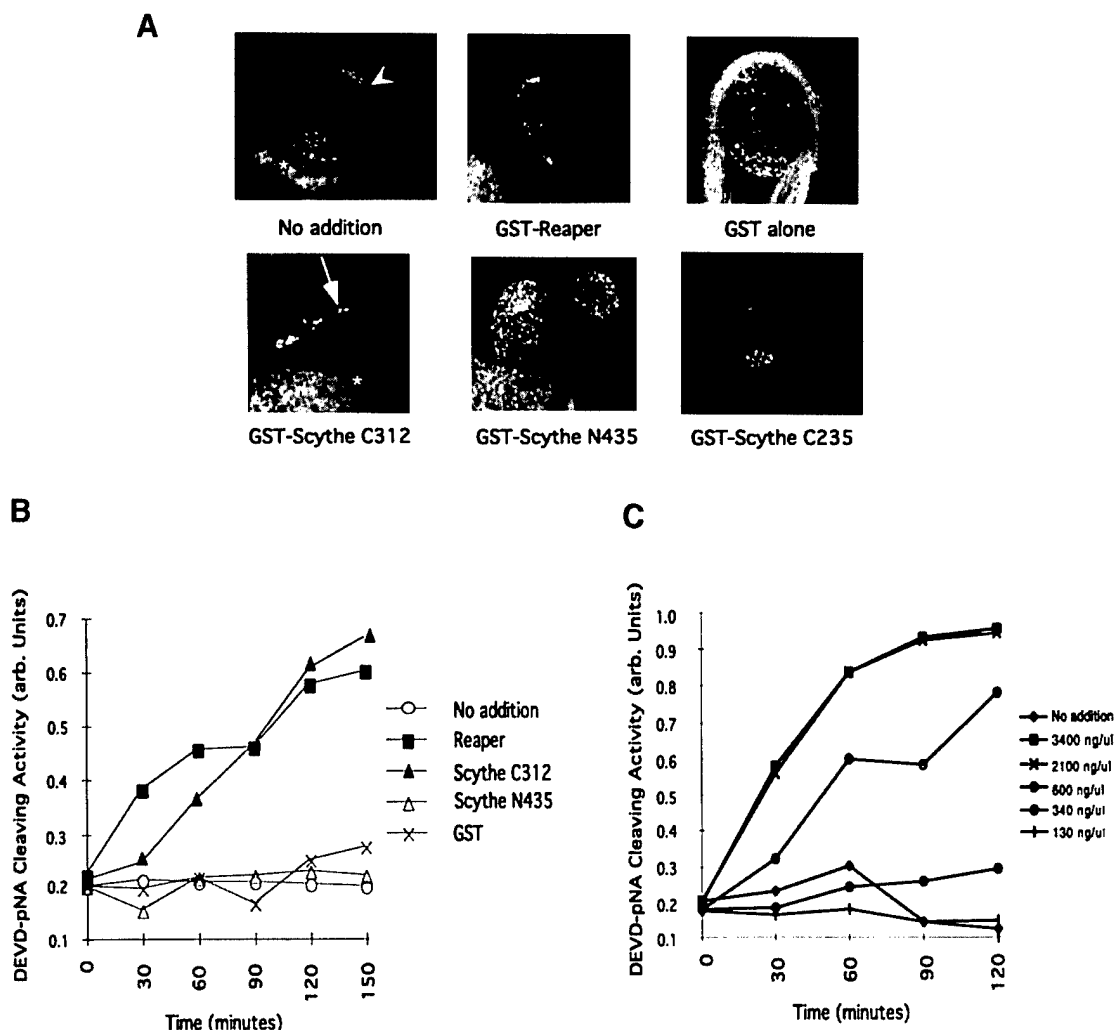
A

| No addition | GST-Reaper | GST alone |

| GST-Scythe C312 | GST-Scythe N435 | GST-Scythe C235 |

B

Time (minutes)

C

Time (minutes)

Fig. 2. ScytheC312 induces morphological characteristics of apoptosis and DEVDase activation. The indicated GST fusion proteins (final concentration 600 ng/μl) or an equivalent amount of GST protein alone were added to *Xenopus* egg extract in the presence of sperm chromatin to form synthetic nuclei in the extracts (1000 nuclei/μl) and an ATP regeneration system. (A) Photomicrographs of representative nuclei upon staining with the DNA intercalating dye, Hoescht 33258, 90 min after protein addition: dotted arrow = uncondensed interphase chromatin contained within an intact nuclear envelope, solid arrow = condensed, apoptotically fragmented chromatin, * = background staining of membranes present in the extract. (B) At the indicated times, 2 μl of extract were collected for a DEVD-pNA cleavage assay. (C) The indicated amounts of ScytheC312 were added to extracts and, at the indicated times, 2 μl of extract were collected for a DEVD-pNA cleavage assay.

activation, reaper-induced mitochondrial cytochrome *c* release (Figure 5B and C) and reaper-induced apoptotic nuclear fragmentation (data not shown). However, DEVDase activity was still induced in depleted extracts upon addition of recombinant caspase 8, at concentrations of caspase 8 which exhibited no intrinsic DEVDase activity (Figure 5D). In addition, Scythe-depleted extracts manifested all of the characteristic morphological changes of apoptosis upon addition of caspase 8, again showing that these extracts were still responsive to previously activated caspases (data not shown). Moreover, ScytheC312 was still able to induce apoptosis in extracts immunodepleted of full-length Scythe, indicating that Scythe-responsive factors were still active in the extract (Figure 6). These data demonstrate that Scythe is an essential intermediate in the reaper-induced apoptotic pathway.

ScytheC312-interacting factors are required for reaper-induced apoptosis

The ability of ScytheC312 to induce apoptosis in extracts depleted of full-length Scythe suggests that pro-apoptotic factors engaged by ScytheC312 remain in the extract following Scythe removal. If such factors are *bona fide* signaling components in reaper-induced apoptosis, then their removal should block reaper-induced apoptosis even in the presence of Scythe. To explore this issue, we coupled ScytheC312 or ScytheN435 to Sepharose beads to produce a resin capable of depleting Scythe-interacting factors from extracts. These Scythe 'beads' were incubated in extracts and then removed by gentle centrifugation. The depleted extracts were then incubated with reaper protein. We found that depletion of ScytheC312-interacting factors from the extracts blocked reaper-induced DEVDase activation, though recombinant caspase 8 was still effective
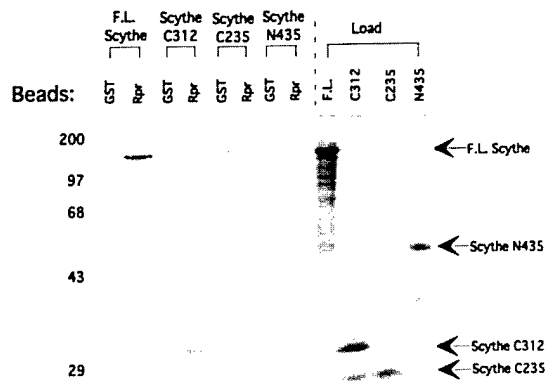
Fig. 3. ScytheC312, but not ScytheN435 or C235, interacts with reaper. [35]S-Labeled full-length (F.L.) Scythe, ScytheC312, C235 and N435 proteins were incubated with GST or GST–reaper beads in the presence of *Xenopus* egg extract for 60 min at room temperature. Beads were then washed three times with ELB, resolved by SDS–PAGE and processed for autoradiography. If 100% of the input [35]S-labeled proteins were bound to reaper, the intensity of the signal would be equivalent to that seen in the control 'Load' lanes. Note that 100% recovery is unlikely due to competition from endogenous Scythe present in the extract.
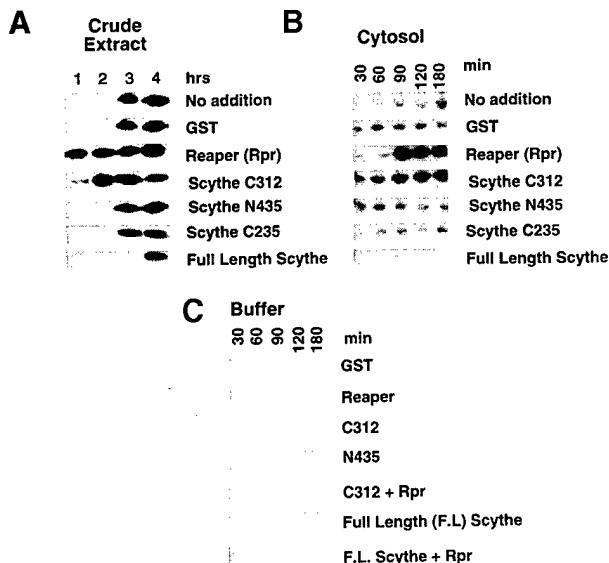

Fig. 4. ScytheC312 accelerates cytochrome *c* release from mitochondria. Recombinant GST protein or the indicated GST fusion proteins were added to either (**A**) crude egg extract, (**B**) mitochondria and egg cytosol, or (**C**) buffer alone. At the indicated times, the samples were filtered through a 0.1 μM microfilter to remove particulate components, including mitochondria. Aliquots (10 μl) of protein filtrate were separated by SDS–PAGE and processed for Western blot with an anti-cytochrome *c* monoclonal antibody. Note that there is extract to extract variability in the absolute timing of cytochrome *c* release. The cytosol in (B) is not from the same batch of *Xenopus* eggs as the crude extract used in (A). Hence, the absolute time course of cytochrome *c* release is slightly different in these two panels.

at inducing DEVDase activity and apoptotic nuclear fragmentation in these extracts (Figure 7 and data not shown). In contrast, depletion of ScytheN435-interacting factors had no effect (Figure 7). These data show that factors that act downstream of ScytheC312 are critical for reaper-induced apoptosis.

Collectively, our data suggest that reaper activation of Scythe promotes a conformational change which can be

mimicked by truncation of Scythe. Pro-apoptotic factors do not appear to bind to Scythe prior to reaper addition (since they remain in the extract after immunodepletion of endogenous Scythe), but the conformational change leads to their engagement and, ultimately, to mitochondrial cytochrome *c* release and cell death.

Discussion

This report describes the isolation and characterization of a novel apoptotic regulator, Scythe, which binds tightly to reaper, a central regulator of programmed cell death in *Drosophila*. The ability of recombinant reaper to induce apoptosis in *Xenopus* egg extracts provided the first evidence that reaper could engage the apoptotic machinery in vertebrate cells. Here we show that Scythe is a critical component of this reaper-responsive machinery.

Sequence features of Scythe

The primary structure of the Scythe protein is rather unremarkable. Only the N-terminal 80 amino acids, which are 54% similar to ubiquitin, bear homology to other reported protein sequences. Recently, it was reported that sentrin, a protein of 100 amino acids with 48% similarity to ubiquitin, can interact in the yeast two-hybrid system with the intracellular domains of two potent apoptotic regulators, Fas/APO-/CD95 and the tumor necrosis factor (TNF) receptor (Okura *et al.*, 1996). While it remains to be seen whether the N-terminal region of Scythe serves to link Scythe to other apoptotic regulators, we found that depletion of extracts on a resin linked to the N-terminus of Scythe did not disrupt the ability of reaper to induce apoptosis. Moreover, the reaper-binding site on Scythe lies within the C-terminal 312 amino acids of Scythe.

We noted within the primary sequence of Scythe at least one potential caspase cleavage site, DDVD, beginning at amino acid 832. Although Scythe could be cleaved at multiple sites *in vitro*, we were unable to detect any cleavage of the endogenous Scythe protein in reaper-treated egg extracts with high levels of DEVDase activity (data not shown). However, it may be that other factors in the full extract protect Scythe from cleavage.

The Scythe protein bound to reaper migrates as a 148/150 kDa doublet on SDS–polyacrylamide gels. Because *Xenopus* is pseudo-tetraploid, we suspect that these bands represent closely related, though not identical gene products. Indeed, all of the peptide sequences obtained from both protein species were identical to sequences encoded by our cloned Scythe cDNA. *In vitro* translation of this Scythe-encoding cDNA does not produce two forms of the protein, nor do additional forms appear after incubation of the translated product in *Xenopus* egg extracts. This suggests the possibility that the electrophoretic mobility shift may not have been due to post-translational modification.

Truncation of Scythe mimics binding by reaper

We found that recombinant full-length Scythe produced in baculovirus-infected Sf9 cells retained reaper-binding ability (data not shown), but did not induce detectable caspase activation or nuclear fragmentation upon addition to *Xenopus* egg extracts. There are at least two possible interpretations of these data (in addition to the trivial
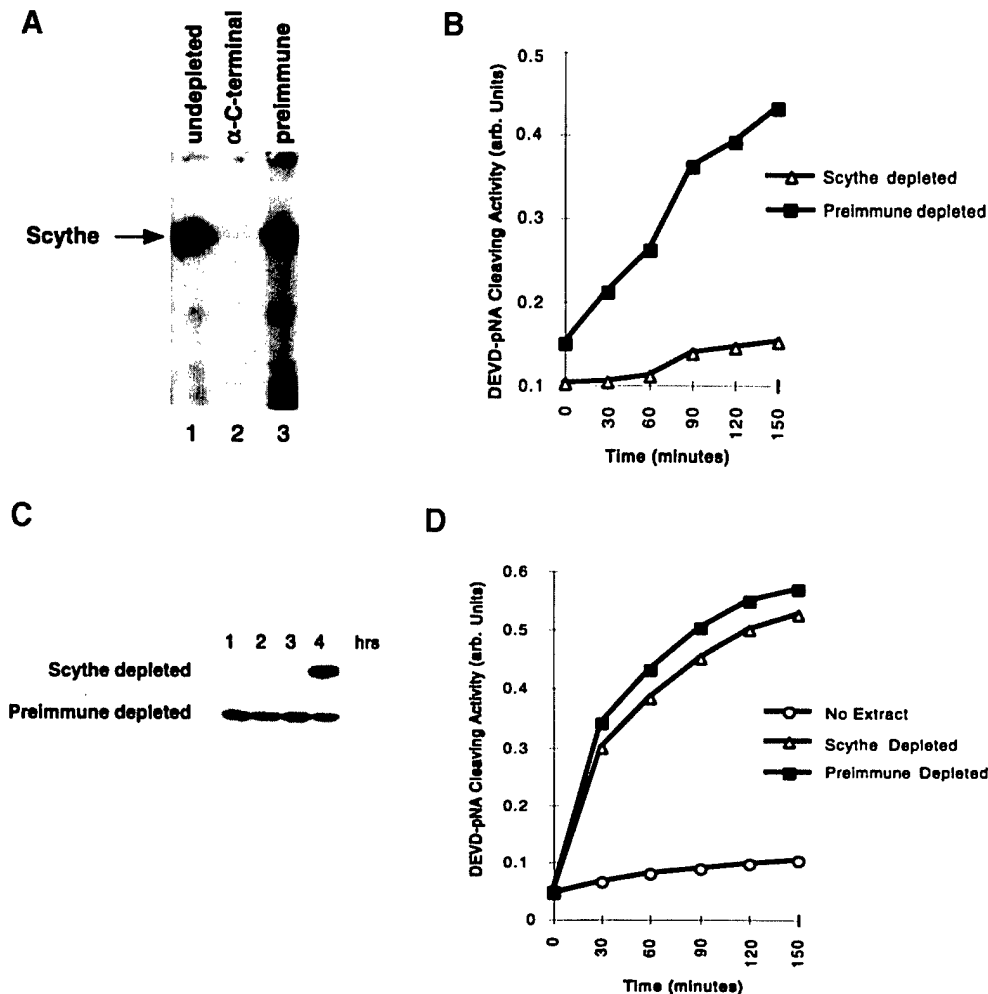
Fig. 5. Depletion of Scythe inhibits reaper-induced DEVDase activation and mitochondrial cytochrome *c* release. (**A**) Scythe was immunodepleted from 100 μl of crude extract using anti-ScytheC312 sera linked to protein A–Sepharose. After three successive rounds of immunodepletion, 10 μl aliquots of extract were resolved by SDS–PAGE and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 40 amino acids of the *Xenopus* Scythe protein. Lane 1, undepleted extract; lane 2, extract depleted with anti-Scythe C312 sera; lane 3, extract depleted with pre-immune sera. (**B**) Recombinant reaper protein (600 ng/μl) was added to either extract depleted of endogenous Scythe protein or extracts similarly treated with pre-immune sera. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (**C**) Recombinant reaper protein (600 ng/μl) was added to either Scythe-depleted or pre-immune-depleted *Xenopus* egg extracts. At the indicated times, the samples were filtered through a 0.1 μM microfilter to remove particulate components, including mitochondria. Aliquots (10 μl) of cytosolic protein were separated by SDS–PAGE and processed for Western blot with an anti-cytochrome *c* monoclonal antibody. (**D**) Recombinant, active caspase 8 (lacking the pro-domain: 400 ng/μl) was added to buffer (no extract), extract depleted of endogenous Scythe protein or extracts similarly treated with pre-immune sera. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity.

possibility that the full-length recombinant protein does not fold properly). Conceivably, insect cells produce an anti-apoptotic factor that associates with Scythe and blocks its action (although a stoichiometric inhibitor would have to be present at very high levels). Alternatively, full-length Scythe may not be pro-apoptotic *per se*. This idea is consistent with the fact that the extracts contain abundant Scythe yet require reaper addition to become rapidly apoptotic. Moreover, the observation that full-length Scythe had some suppressive effects on cytochrome *c* release suggests that full-length Scythe may have some anti-apoptotic activity prior to engagement by reaper. Reaper binding might alter the conformation of Scythe, allowing it to activate downstream pro-apoptotic effectors. Indeed, ScytheC312 (the C-terminal 312 amino acids) induced apoptosis in the absence of reaper. Depletion of ScytheC312-interacting factors prevented reaper-induced apoptosis, strongly supporting the notion that the pro-

apoptotic pathway engaged by reaper is identical to that engaged by ScytheC312. We propose that reaper binding allows an otherwise masked Scythe C-terminus to interact with downstream apoptotic regulators. Whether the C-terminus is normally masked by an N-terminal portion of Scythe or by other cellular factors merits future investigation. Alternatively, if Scythe has intrinsic anti-apoptotic activity, then Scythe C312 may be acting as a trans-dominant inhibitor of the endogenous Scythe protein.

Scythe is an indirect inducer of mitochondrial cytochrome c release

The requirement for accessory factors in order for reaper to induce mitochondrial cytochrome *c* release provided the impetus to search for factors which might lie between reaper and the mitochondria. Since immunodepletion of Scythe from cytosol prevented reaper-induced mitochondrial cytochrome *c* release, Scythe is undoubtedly one
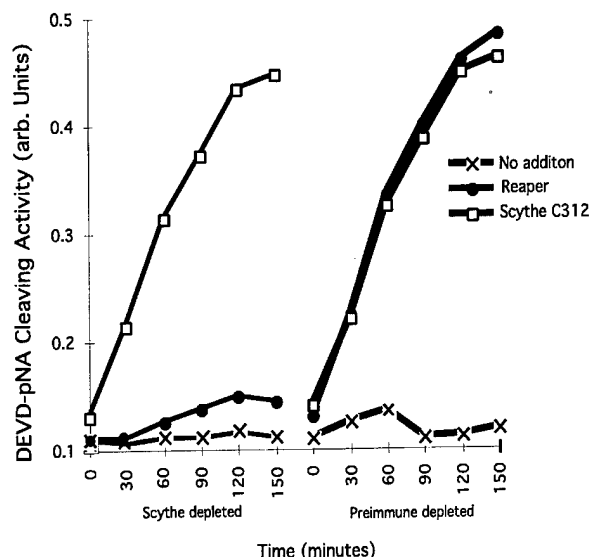
Fig. 6. The C-terminal 312 amino acids of Scythe are capable of inducing DEVDase activation in extracts depleted of endogenous Scythe protein. Recombinant reaper protein (600 ng/µl), an equivalent amount of recombinant ScytheC312 protein, or buffer was added to either Scythe-depleted or pre-immune-depleted extracts and, at the indicated times, 2 µl aliquots of extract were processed for DEVD-pNA cleavage activity.
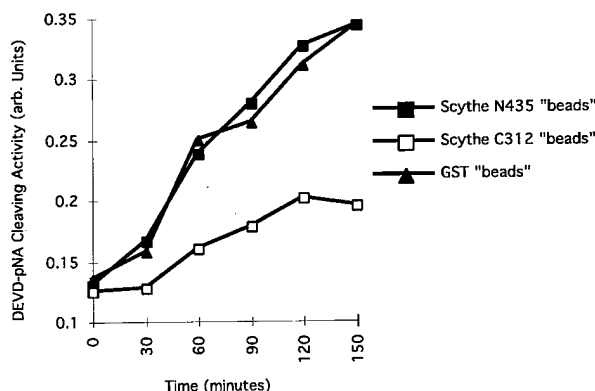

Fig. 7. Depletion of ScytheC312-interacting factors inhibits reaper-induced DEVDase activation. Recombinant reaper was added to *Xenopus* egg extract that had been depleted with the indicated 'beads'. At the indicated times, 2 µl aliquots of extract were collected for a DEVD-pNA cleavage assay.

such factor. However, Scythe is unlikely to be a direct cytochrome c-releasing factor, because neither ScytheC312 nor full-length Scythe bound to recombinant reaper could induce cytochrome c release in the absence of cytosolic factors. Since depletion of extracts using a ScytheC312 resin abrogated reaper-induced apoptosis, it is likely that at least one of the factors required for reaper-induced cytochrome c release can physically interact with the C-terminal region of Scythe.

Is there a vertebrate reaper?

Efforts by our group and others have failed to identify a vertebrate reaper homolog using standard molecular cloning techniques. Given our findings, we hypothesize that *Drosophila* reaper triggers apoptosis in *Xenopus* egg extracts by mimicking an endogenous vertebrate Scythe-activating factor. By analogy to reaper, such a Scythe-

activating factor might be transcriptionally induced in response to external stimuli or in response to developmental cues. Therefore, using Scythe as a 'bait' to search for reaper-like factors in extracts from appropriately staged or irradiated embryos may provide a means to isolate reaper-like factors which may not be well conserved at the primary sequence level. It will be equally interesting to determine whether there are Scythe-related proteins acting downstream of reaper in *Drosophila*. It is theoretically possible that reaper accesses an apoptotic pathway in *Xenopus* egg extracts which is distinct from that used in flies.

Using recombinant reaper, we have uncovered a novel component of a reaper-responsive apoptotic signaling pathway which also has the ability, upon truncation, to trigger apoptosis independently. Taken together, our data suggest that Scythe is a critical link between reaper and downstream factors required for mitochondrial cytochrome c release. We anticipate that Scythe will provide a foothold not only into the isolation of vertebrate reaper-like factors, but also into the pathway leading from reaper to the mitochondria and apoptotic cell death.

Materials and methods

Preparation of Xenopus egg extracts

For induction of egg laying, mature female frogs were injected with 100 U of pregnant mare serum gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (HCG; USB). At 14–20 h after injection with HCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine (pH 7.8), washed three times in modified Ringer's solution (MMR) (1 M NaCl, 20 mM KCl, 10 mM MgSO$_4$, 25 mM CaCl$_2$, 5 mM HEPES pH 7.8, 0.8 mM EDTA) and then washed in egg lysis buffer [ELB; 250 mM sucrose, 2.5 mM MgCl$_2$, 1.0 mM dithiothreitol (DTT), 50 mM KCl, 10 mM HEPES] pH 7.4. Eggs were packed by low-speed centrifugation at 400 g. Following addition of aprotinin and leupeptin (final concentration 5 µg/ml), cytochalasin B (final concentration 5 µg/ml) and cycloheximide (final concentration 50 µg/ml), eggs were lysed by centrifugation at 10 000 g for 15 min. For nuclear formation, extracts were supplemented with demembranated sperm chromatin (1000 nuclei/µl) and an ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP and 50 µg/ml creatine phosphokinase). In some experiments, extracts were treated with recombinant GST–reaper protein, GST–Scythe protein and/or baculovirus-expressed His-tagged full-length Scythe protein (all at a final concentration of 600 ng/µl). For assessment of apoptotic nuclear morphology after Scythe or reaper addition, samples were withdrawn at regular intervals during room temperature incubation and visualized by fluorescence microscopy following staining with Hoechst 33258 and formaldehyde fixation. In assays measuring caspase cleavage of [35]S-labeled Scythe, extracts were supplemented with 1/10 volume of rabbit reticulocyte lysate containing [35]S-labeled Scythe in the presence or absence of recombinant reaper protein. Samples were then diluted with 2× sample buffer, resolved by SDS–PAGE and processed for autoradiography.

Protein biotinylation

GST or GST–reaper protein coupled to glutathione–Sepharose beads were washed three times with ELB. The GST–protein beads were then blocked by incubation with 10 mg/ml bovine serum albumin (BSA; fraction V) in ELB for 30 min at 4°C. The bead–protein complex was then pelleted and washed twice with ELB. Crude extract was then added at 10 times the volume of beads and rotated at 4°C for 90 min. The beads were then pelleted, washed twice in ELB, twice in sodium bicarbonate buffer (NaBicarb), resuspended in 1 ml of NaBicarb and incubated with 30 µl of biotinylation reagent (Amersham Biotinylation module) for 1 h at room temperature. The beads were again pelleted, washed three times with ELB and diluted with 2× sample buffer. The samples were then resolved by SDS–PAGE, transferred to PVDF

immobilon, probed with HRP-linked streptavidin and visualized through an ECL chemiluminescence detection system.

Protein sequencing of Scythe

Starting with 15 ml of crude *Xenopus* extract and ~700 µg of GST–reaper protein as 'bait', ~2–3 pmol of the 148/150 kDa doublet were obtained using the bead-binding protocol outline above. After the proteins were separated on an SDS–PAGE gel, they were electroblotted onto a PVDF membrane (ProBlott, Applied Biosytem) and visualized by staining with 0.1% Coomassie Blue in 50% methanol. After excision of the 148/150 kDa bands from the membrane, the bands were reduced and alkylated with isopropylacetamide followed by digestion in 20 µl of 0.05 M ammonium bicarbonate containing 0.5% Zwitergent 3–16 (Calbiochem) with 0.2 µg of trypsin (Frozen Promega Modified) or Lysine-C (Wako) at 37°C for 17 h as described previously (Krutzsch and Inman, 1993; Lui *et al.*, 1996). The solution was then injected directly onto a 0.32×150 mm C18 capillary column. Peptides generated from *in situ* digests were separated on a C18 0.32×100 mm capillary column (LC Packing, Inc.). The HPLC consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 UV detector equipped with a Z-shaped flow cell (LC Packing, Inc.). A 30 cm length of 0.025 mm i.d. glass capillary was connected to the outlet of the Z-shaped cell inside the detector housing to minimize the delay volume. The total delay volume was 0.45 µl which corresponded to a delay of 6 s for a flow rate of 3.5 µl/min. The short delay greatly facilitated hand collections of the HPLC fractions (Henzel and Stults, 1995). Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.08% TFA. The peptides were eluted with a linear gradient of 0–80% B in 60 min, detected at 195 nm and hand collected into 0.5 ml Eppendorf tubes.

An aliquot (0.2 µl) of each of the isolated HPLC fractions was applied to a pre-made spot of matrix (0.5 µl of 20 mg/ml α-cyano-4-hydroxycinammic acid plus 5 mg/ml nitrocellulose in 50% acetone/50% 2-propanol) on the target plate (Shevchenko *et al.*, 1996). Ions were formed by matrix-assisted laser desorption/ionization with a 337 nm nitrogen laser. Spectra were acquired with a PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer, operated in liner delayed extraction mode. Subsequently, fragment ions for selected precursor masses were obtained from postsource decay (PSD) experiments (Kaufmann *et al.*, 1994). To enhance the ion abundances at low mass, collision gas (air) was introduced to the collision cell during the acquisition of the lower portion of the fragment ion spectrum. Each peptide mass and its associated fragment ion masses was used to search an in-house sequence database with an enhanced version of the FRAGFIT program (Henzel *et al.*, 1993). The program was modified to permit potential methionine oxidation and partial proteolytic cleavage. Furthermore, experimentally determined PSD fragment ion masses can be compared with theoretical fragment ions (b and y ions) from the database entries (Clauser *et al.*, 1995). The latter approach provides a high degree of searching specificity without the need for spectral interpretation. Peptide fractions were sequenced on a model 494CL PE Applied Biosystems sequencer using 6 mm microcartridges and equipped with an on-line parathyroid (PTH) analyzer (model 140D). Peaks were integrated with Justice innovation software using Nelson Analytical 760 interfaces. Sequence intepretation was perfromed on a DEC Alpha computer (Henzel *et al.*, 1987).

cDNA cloning of Scythe

A 535 bp *SacI–EcoRI* fragment of the human BAT3 cDNA (a generous gift from Dr Thomas Spies) was labeled with [α-³²P]dCTP using the Random Primed DNA labeling kit (Boehringer Mannheim). This cDNA fragment was then used to screen a λZAP *Xenopus* library (gift from Dr Bruce Mayer) by hybridizing duplicate filters at 37°C overnight. The filters were washed twice with 2× SSC/0.1% SDS at 37°C for 30 min and twice more with 1× SSC/0.1% SDS at 42°C for 30 min. Of the 5×10⁵ plaques screened, six positive clones were identified. After rescue, three of these clones were found to be ~3.8 kb in length. These three clones were sequenced and found to represent the full-length *Xenopus* cDNA homologs of BAT3.

Preparation of GST–Scythe protein

Three separate truncations of recombinant *Xenopus* Scythe protein were constructed: the C-terminal 312 amino acids, C-terminal 235 amino acids and N-terminal 435 amino acids. cDNA encoding these truncations were PCR amplified using the following primers: C-terminal 312 amino acids, 5′-gat cgg atc cag ctt tgc ctc cgt tac tgt c-3′ and 5′-gat caa gct ttt agg ggt ccc ccc tga a-3′; C-terminal 235 amino acids, 5′-gat cgg atc

cat tcg aag gct ctc tct tga g-3′ and 5′-gat caa gct ttt agg ggt ccc ccc tga a-3′; N-terminal 435 amino acids, 5′-gat cgg atc cat ggc agc taa tgag aaa-3′ and 5′-gat caa gct ttt aag gtc cac cag atg c-3′. PCR fragments were cloned into the expression vector Gex KG, a derivative of Gex 2T (Pharmacia) containing additional polylinker sites and a polyglycine insert, and transformed into the Topp 1 bacterial strain (Stratagene). Recombinant protein was produced as previously described (Evans *et al.*, 1997).

Baculovirus production of full-length Scythe protein

Full-length *Xenopus* Scythe protein was produced using the Bac-to-Bac Baculovirus Expression System (Gibco). Briefly, full-length Scythe was PCR amplified, digested with *NcoI* and *XbaI*, and ligated into the pFastBac vector which had been cut previously with the same enzymes. The resulting donor plasmid encoding an N-terminal 6× His tag preceding full-length Scythe was transformed into DH10Bac *Eschericha coli* cells. These *E.coli* cells containing recombinant bacmid were cultured and recombinant bacmid DNA was recovered using a standard miniprep protocol. Sf-9 insect cells were transfected with the bacmid DNA using CellFECTIN reagent (Gibco), incubated for 48 h at 27°C, and the resulting recombinant baculovirus particles were harvested. Subsequently, Sf-9 cells (2×10⁶ cells/ml) were infected with baculovirus for 48 h, washed twice in phosphate-buffered saline (PBS) and lysed by dounce homogenization in HBS [10 mM HEPES pH 7.5, 20 mM β-glycerolphosphate, 150 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml each of pepstatin, chymostatin and leupeptin]. The lysate was then centrifuged at 4°C for 10 min at 10 000 r.p.m., and the supernatant was incubated with 1 ml of Ni-NTA agarose (Qiagen) for 30 min at 4°C. The beads were washed in 50 volumes of HBS and eluted with HBS containing 200 mM imidazole in five fractions of 500 µl each.

Cytochrome c release assays

To fractionate the crude egg extract into cytosolic and membranous components, the crude extract was centrifuged further at 55 000 r.p.m. (200 000 *g*) in a Beckman TLS-55 rotor for the TL-100 centrifuge for 1 h. The cytosolic and heavy membrane fractions (enriched in mitochondria) were removed, and the cytosolic fraction was re-centrifuged at 55 000 r.p.m. for an additional 25 min. The mitochondrial fraction was purified further by centrifugation of the heavy membrane through a Percoll gradient consisting of 42, 37, 30 and 25% Percoll in mitochondria isolation buffer (1 M sucrose, 100 mM ADP, 2.5 M KCl, 1 M DTT, 1 M succinate, 1 M HEPES–KOH pH 7.5, 0.5 M EGTA, 1.5 M mannitol) for 25 min at 25 000 r.p.m. with no brake in the TLS-55 rotor. The isolated heavy membrane fraction containing mitochondria was diluted 1:10 into cytosol or ELB containing an ATP-regenerating cocktail (10 mM phosphocreatine, 2 mM ATP and 50 µg/ml creatine phosphokinase). At various time points, the cytochrome *c* content was analyzed after filtering 25 µl of the mixture through a 0.1 µm ultrafree-MC filter (Millipore). Aliquots of 10 µl of cytosolic protein were then separated by SDS–PAGE and immunoblotted with an anti-cytochrome *c* monoclonal antibody (Pharmingen), HRP-linked anti-mouse sera and an ECL chemiluminescence detection system (Amersham).

DEVDase assays

To measure caspase activity, 3 µl of each sample were incubated with 90 µl of assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric substrate Ac-DEVD-pNA (final concentration, 200 µM: Biomol Caspase-3 assay system) at 37°C. At various time points, absorbance was measured at 405 nm in a LabSystems MultiSkan MS microtiter plate reader.

Immunodepletion assays

Protein A–Sepharose beads were washed in ELB and pre-incubated with 10 mg/ml BSA in ELB for 40 min at 4°C. The beads were washed twice more with ELB, and 10 µl of Sepharose beads were incubated with 100 µl of pre-immune or anti-Scythe antisera at 4°C for 70 min. The beads were washed again with ELB and then incubated with 100 µl of either the crude *Xenopus* egg extract or isolated cytosol. After 1 h at 4°C, the antibody–bead complexes were pelleted, the supernatant was transferred to a fresh microfuge tube and the depletion process was repeated, using fresh beads, twice more. This depleted extract was then assayed for the ability to induce apoptotic nuclear fragmentation, cytochrome *c* release or DEVDase activation.

Acknowledgements

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Reaper-induced dissociation of a Scythe-sequestered cytochrome c-releasing activity

Kenneth Thress, Erica K.Evans and Sally Kornbluth[1]

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Box 3686, C366 LSRC, Research Drive, Durham, NC 27710, USA

[1]Corresponding author
e-mail: kornb001@mc.duke.edu

Reaper is a potent apoptotic inducer critical for programmed cell death in the fly *Drosophila melanogaster*. While Reaper homologs from other species have not yet been reported, ectopic expression of Reaper in cells of vertebrate origin can also trigger apoptosis, suggesting that Reaper-responsive pathways are likely to be conserved. We recently reported that Reaper-induced mitochondrial cytochrome c release and caspase activation in a cell-free extract of *Xenopus* eggs requires the presence of a 150 kDa Reaper-binding protein, Scythe. We now show that Reaper binding to Scythe causes Scythe to release a sequestered apoptotic inducer. Upon release, the Scythe-sequestered factor(s) is sufficient to induce cytochrome c release from purified mitochondria. Moreover, addition of excess Scythe to egg extracts impedes Reaper-induced apoptosis, most likely through rebinding of the released factors. In addition to Reaper, Scythe binds two other *Drosophila* apoptotic regulators: Grim and Hid. Surprisingly, however, the region of Reaper which is detectably homologous to Grim and Hid is dispensable for Scythe binding.
Keywords: apoptosis/cytochrome c-releasing activity/Reaper/Scythe

Introduction

Apoptosis is a program of cellular suicide, which leads to the elimination of excess or damaged cells while leaving neighboring cells unperturbed. Apoptosis is critical for organismal homeostasis in the adult and is an integral part of the developmental program in all metazoans (Vaux *et al.*, 1994; Steller, 1995).

With a few exceptions, cellular death by apoptosis is executed by a family of aspartate-directed cysteine proteases known as the caspases (Chinnaiyan and Dixit, 1996). These enzymes, responsible for cleaving a battery of proteins during apoptotic cellular execution, are synthesized as inactive zymogens. Activation of pro-caspases can be triggered by binding of regulatory proteins to their pro-domains (most likely inducing pro-caspase oligomerization) or through cleavage *in trans* by already activated caspases (Muzio *et al.*, 1998; Yang *et al.*, 1998). In many instances, apoptotic pathways leading to caspase activation proceed via signaling-induced release of cytochrome c from the intermembrane space of the mito-

chondria to the cytosol. In the cytosol, cytochrome c serves as a cofactor, with the protein Apaf-1, to activate pro-caspase 9. Active caspase 9 then activates other caspases, most notably one of the prominent effector caspases, caspase 3 (Liu *et al.*, 1996; Kluck *et al.*, 1997; Zou *et al.*, 1997).

The activation of caspases and, ultimately, apoptosis can be blocked by members of several different protein families. Those characterized most well are the IAP (inhibitor of apoptosis) proteins and anti-apoptotic members of the Bcl-2 family (Adams and Cory, 1998; Deveraux and Reed, 1999). A growing number of proteins in the Bcl-2 family can modulate apoptosis either positively or negatively. At least in vertebrate cells, it appears that the primary locus of Bcl-2 family action is the mitochondrion. Bcl-2 and its relative Bcl-xL can suppress mitochondrial cytochrome c release, while several of the pro-apoptotic Bcl-2 family members, including Bid, Bax and Bak, can accelerate its release (Li *et al.*, 1998; Luo *et al.*, 1998; Desagher *et al.*, 1999; Griffiths *et al.*, 1999; Gross *et al.*, 1999). Interestingly, it was recently reported that Bcl-2 family members can bind to the mitochondrial voltage-dependent anion channel to modulate cytochrome c release (Shimizu *et al.*, 1999).

IAPs were first described as baculoviral proteins involved in the suppression of virally induced host cell death (Crook *et al.*, 1993; Birnbaum *et al.*, 1994; Clem and Miller, 1994). Subsequently, it has been shown that cellular IAPs exist in a number of species examined, and human IAPs Xiap, c-Iap1 and c-Iap2 can all prevent pro-caspase activation. Baculoviral IAP repeat (BIR) domains present in all of the IAPs are necessary, and in some cases sufficient, to suppress caspase activation and apoptosis (Roy *et al.*, 1997; Deveraux *et al.*, 1998; Takahashi *et al.*, 1998). The precise molecular mechanism of this suppression is not yet understood.

Genetic analysis in several organisms has successfully identified novel apoptotic regulators which, acting in conjunction with proteins such as IAPs, caspases and Bcl-2 family members, are critical for implementation of the cell death program. In an extensive analysis of chromosomal deletion mutants in the fly *Drosophila melanogaster*, Steller and colleagues identified a chromosomal region containing a number of genes critical for programmed cell death occurring during embryonic development (White *et al.*, 1994). Three genes in this region encode Reaper, Hid and Grim proteins, which are potent cell death inducers (Grether *et al.*, 1995; Chen *et al.*, 1996b; White *et al.*, 1996). In their absence, cell death is abrogated, while ectopic expression of these genes promotes apoptotic death not only in fly cells, but in human cells as well (Claveria *et al.*, 1998; McCarthy and Dixit, 1998; Haining *et al.*, 1999).

In an effort to understand the mechanism of action of

Reaper protein, we produced recombinant Reaper and examined its effects in cell-free extracts prepared from *Xenopus* eggs. While these extracts will spontaneously release mitochondrial cytochrome *c* and activate endogenous caspases after prolonged incubation at room temperature (~4.5–7 h), Reaper addition greatly accelerated this process, triggering mitochondrial cytochrome *c* release, caspase activation and fragmentation of added nuclei within ~1.5–2 h (Newmeyer *et al.*, 1994; Evans *et al.*, 1997a,b).

As Reaper is a very small protein (65 amino acids) with no significant homology to known signaling molecules and no evident catalytic function, we searched for Reaper-interacting molecules potentially required for Reaper-induced apoptosis. In doing so, we identified a 150 kDa Reaper-binding protein, which was named Scythe (Thress *et al.*, 1998). While the primary amino acid sequence of Scythe did not provide any clues as to its mechanism of action, immunodepletion of Scythe from *Xenopus* egg extracts eliminated Reaper-induced cytochrome *c* release, caspase activation and the induction of apoptotic nuclear fragmentation. Moreover, a truncated variant of Scythe (Scythe C312), consisting of the C-terminal 312 amino acids of Scythe fused to glutathione *S*-transferase (GST), induced apoptosis in the egg extracts very effectively, even in the absence of Reaper. Scythe C312 protein induced mitochondrial cytochrome *c* release in *Xenopus* egg extracts, but could not trigger cytochrome *c* release from purified mitochondria, indicating a requirement for accessory cytosolic factors. Collectively, these experiments led to the conclusion that Scythe was a novel apoptotic regulator required in the pathway of Reaper-induced apoptosis, but that additional factors were required to promote Reaper-induced cytochrome *c* release and consequent caspase activation.

In this report, we demonstrate that Scythe sequesters positive regulators of apoptosis that, when not bound by Scythe, can trigger cytochrome *c* release from purified mitochondria in the absence of other cytosolic components. Importantly, we show that this cytochrome *c*-releasing activity is liberated when Reaper binds to Scythe, providing a mechanistic explanation for the Scythe requirement in Reaper-induced apoptosis.

Results

Excess Scythe inhibits Reaper-induced apoptosis

We previously reported that Scythe C312 could induce mitochondrial cytochrome *c* release and consequent caspase activation upon addition to egg extracts. According to this scenario, Scythe C312 was an 'activated' Reaper-independent Scythe variant, mimicking a conformation characteristic of Reaper-bound Scythe. Indeed, addition of excess recombinant Scythe on its own never triggered apoptosis, suggesting that this excess Scythe could not adopt an activated C312-like pro-apoptotic conformation in the absence of Reaper. Nonetheless, we reasoned that co-addition of Reaper along with excess full-length Scythe might produce a particularly robust apoptotic response, perhaps leading to swifter or greater caspase activation than that induced by Reaper alone. To test this, we added 300 ng/µl exogenous Scythe protein (~5-fold that found endogenously) to *Xenopus* egg extracts along with recom-
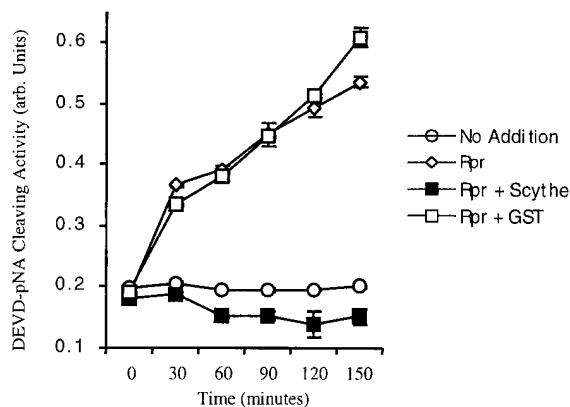

Fig. 1. Recombinant Scythe inhibits Reaper-induced caspase activity. Recombinant Reaper (Rpr) protein alone (300 ng/µl) or Reaper in combination with equivalent amounts of either recombinant Scythe or GST proteins was added to *Xenopus* egg extracts. At the indicated times, 2 µl aliquots of extract were analyzed for caspase activity using a DEVD-pNA cleavage assay.

binant Reaper and monitored apoptotic progression. Surprisingly, we found that addition of excess full-length Scythe suppressed, rather than accelerated, Reaper-induced caspase activation (Figure 1) and morphological apoptosis (data not shown). These data raised the possibility that Scythe might have intrinsic anti-apoptotic activity that could be antagonized by Reaper. Intriguingly, we found that spontaneous apoptosis following prolonged incubation of egg extracts at room temperature was also inhibited by addition of excess Scythe, reinforcing the hypothesis that Scythe is inherently anti-apoptotic (data not shown). Importantly, excess Scythe was not able to inhibit apoptosis induced by the addition of low levels of recombinant caspase 8 (not shown), demonstrating that excess Scythe did not destroy the competence of the extract to undergo apoptosis.

A Scythe-sequestered apoptotic inducer is released by Reaper

Since immunodepletion of Scythe from egg extracts prevents Reaper-induced apoptosis, but Scythe itself appeared to be anti-apoptotic, we suspected that a pro-apoptotic factor required for Reaper-induced apoptosis might be bound to, and co-depleted with, Scythe. A model for at least one pathway of Reaper-induced apoptosis, based on this hypothesis, is shown in Figure 2A. According to this model, endogenous Scythe in the extract sequesters a pro-apoptotic factor ('X'). Upon binding of Reaper to Scythe, 'X' is released, thereby triggering mitochondrial cytochrome *c* release and consequent caspase activation. This model would account for the inability of full-length Scythe to induce apoptosis, as well as the observation that excess Scythe could inhibit Reaper-induced apoptosis; after Reaper-induced release of 'X' from a subpopulation of Scythe, excess Scythe would simply resequester 'X'. This hypothesis predicts that immunoprecipitates of Scythe should contain a pro-apoptotic factor susceptible to release from the precipitate following addition of Reaper protein.

To test this hypothesis, we linked either anti-Scythe or pre-immune sera to protein A–Sepharose, incubated these 'beads' in crude *Xenopus* egg extract, and then pelleted and washed the beads. The washed precipitates were then
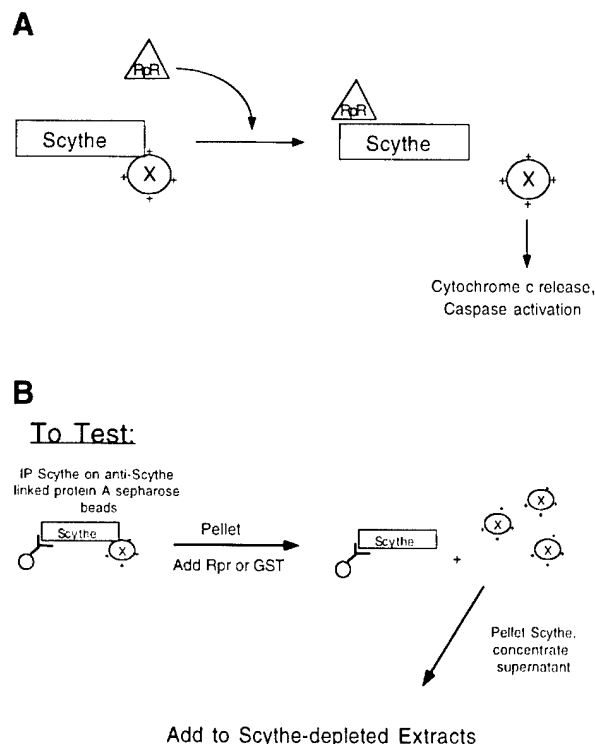
A

B

To Test:

Add to Scythe-depleted Extracts

Fig. 2. A possible model for Rpr-induced apotosis via Scythe. (A) In this model, Scythe is normally bound to and sequesters a pro-apoptotic activity, here denoted as factor 'X'. Upon Reaper (Rpr) addition to the extract, Rpr binds to Scythe, thus causing the release of factor 'X' from Scythe. Factor 'X' is then free to induce cytochrome *c* release and subsequent caspase activation. (B) To test the above model, endogenous Scythe (bound to factor 'X') is immunoprecipitated using Scythe antibody linked to protein A–Sepharose beads. The beads are then pelleted, washed, and incubated with recombinant Reaper protein (Rpr) to induce release of factor 'X' into the supernatant. The beads are spun out to remove Scythe and remaining Scythe-associated proteins, and the factor 'X'-containing supernatant is concentrated and added to Scythe-depleted extracts.

A

B

C

Fig. 3. Reaper-induced release of a pro-apoptotic activity from Scythe. (A) Scythe antisera or pre-immune sera linked to protein A–Sepharose beads were incubated with *Xenopus* egg extracts for 1 h at 4°C. After immunoprecipitation, the beads were washed and resuspended in ELB. The washed beads were then incubated with either recombinant GST or GST–Reaper (Rpr) for 30 min at room temperature. The beads were spun out and the supernatant concentrated ~10-fold by centrifugation in microcon 10s for 20 min at 4°C. The resulting samples were added 1:10 to Scythe-depleted egg extract and, at the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (B) The activity released by Reaper is heat labile. Immunoprecipitates from protein A-linked Scythe antibody (Scythe Ab) were incubated with recombinant Reaper (Rpr) protein as described above. After concentration, the supernatants were either added directly to Scythe-depleted extracts or first incubated at 80°C for 10 min prior to addition to extracts. (C) Apoptotically active supernatant does not contain Scythe protein. Equivalent amounts of the indicated samples were separated by SDS–PAGE, processed for immunoblotting using anti-Scythe sera, and Scythe protein visualized via a chemiluminescence detection kit (Amersham). Pre, samples immunoprecipitated with pre-immune 'beads'; Scythe Ab, samples immunoprecipitated with anti-Scythe 'beads'; GST, supernatant treated with GST protein; Rpr, supernatant treated with Rpr protein. Shown in lanes 1 and 2 are proteins remaining in the extract after immunoprecipitation with pre-immune or immune sera. Lanes 3 and 4 show Scythe present in the immune but not pre-immune precipitate. As seen in lanes 5–8, none of the 'released' supernatants contain detectable Scythe protein.

incubated with either GST protein or with GST–Reaper in order to initiate the release of the presumptive pro-apoptotic factor(s) 'X' into the supernatant (Figure 2B). After removal of the bead-bound material (including Scythe and Reaper) by centrifugation, the residual supernatant was concentrated and added to a crude *Xenopus* egg extract that had been entirely immunodepleted of endogenous Scythe protein. In agreement with the proposed model, both caspase activation (Figure 3A) and mitochondrial cytochrome c release (data not shown) were induced by material released from the Scythe immunoprecipitate by Reaper. GST alone did not induce the release of such an activity from the Scythe immunoprecipitate. In addition, Reaper did not induce the release of pro-apoptotic factors from the pre-immune beads (indicating, as expected, that that any Reaper carried over into the supernatant did not induce apoptosis in the Scythe-depleted extract), nor did GST induce the release of such activity from the Scythe immunoprecipitate. The activity released by Reaper appeared to be heat labile, as incubation of the supernatant at 80°C for 10 min inactivated the pro-apoptotic factor(s) in the supernatant (Figure 3B). More-over, the apoptotically active supernatant obtained when GST–Reaper was added to Scythe precipitates did not contain any detectable Scythe protein (Figure 3C), so it
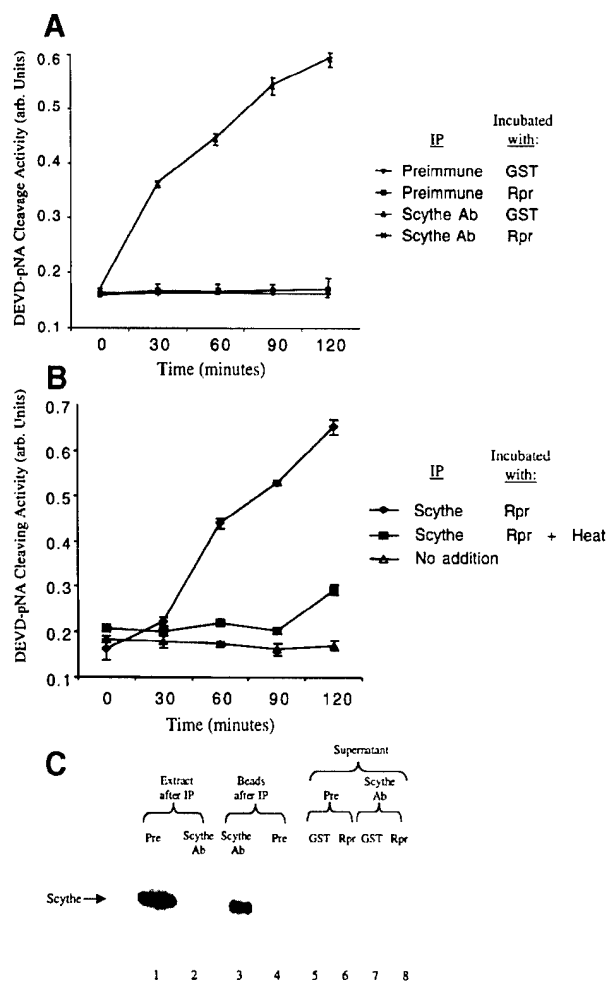
is very unlikely that Scythe–Reaper complexes carried over into the Scythe-depleted extract (see also below).

As described in Figure 1, recombinant Scythe, when present in excess, can inhibit Reaper-induced apoptosis. One potential explanation for this observation is that
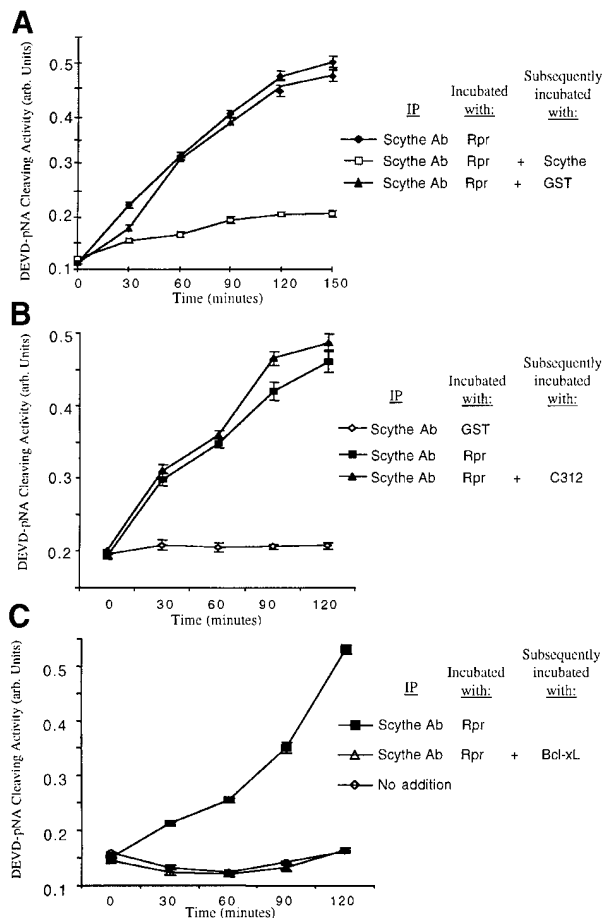
A

DEVD-pNA Cleaving Activity (arb. Units)

0.5
0.4
0.3
0.2
0.1

0 30 60 90 120 150

Time (minutes)

	IP	Incubated with:	Subsequently incubated with:
—●—	Scythe Ab	Rpr	
—□—	Scythe Ab	Rpr	+ Scythe
—▲—	Scythe Ab	Rpr	+ GST

B

DEVD-pNA Cleaving Activity (arb. Units)

0.5
0.4
0.3
0.2

0 30 60 90 120

Time (minutes)

	IP	Incubated with:	Subsequently incubated with:
—◇—	Scythe Ab	GST	
—■—	Scythe Ab	Rpr	
—▲—	Scythe Ab	Rpr	+ C312

C

DEVD-pNA Cleaving Activity (arb. Units)

0.5
0.4
0.3
0.2
0.1

0 30 60 90 120

Time (minutes)

	IP	Incubated with:	Subsequently incubated with:
—■—	Scythe Ab	Rpr	
—△—	Scythe Ab	Rpr	+ Bcl-xL
—◇—	No addition		

Fig. 4. Recombinant Scythe, but not Scythe C312, resequesters the pro-apoptotic activity. (**A**) Immunoprecipitates from protein A-linked Scythe antibody (Scythe Ab) were incubated with recombinant Reaper (Rpr) protein as described above. After concentration, the supernatants were either left untreated or incubated with equivalent amounts of either recombinant Scythe or GST protein for 30 min at 4°C. The resulting samples were added 1:10 to Scythe-depleted egg extract and at the indicated times 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (**B**) An assay identical to (A) was carried out, but instead of incubating the released, concentrated supernatant with recombinant Scythe, the samples were incubated with recombinant Scythe C312 for 30 min at 4°C. (**C**) Recombinant Bcl-xL inhibits released activity. The released, concentrated supernatant was supplemented with recombinant Bcl-xL and added to Scythe-depleted extracts, and at the indicated times 2 μl aliquots of extract were processed for DEVD-pNA cleavage

the exogenously added Scythe can resequester the pro-apoptotic factor(s) released upon Reaper addition. To address this issue, we repeated the release experiments described above, but prior to adding the released proteins to the Scythe-depleted extract, we supplemented the super-natant with either recombinant full-length Scythe or GST protein. As shown in Figure 4A, incubation with Scythe, but not GST, prevented the released supernatant from inducing caspase activation. Collectively, these experiments suggest that Reaper can trigger the release of a pro-apoptotic activity that, once liberated, can initiate cell death through cytochrome *c* release and caspase activation. In contrast to full-length Scythe, the truncated C312 Scythe protein did not suppress the activity of the super-natant released from the Scythe precipitates (Figure 4B).

To characterize further the factors released from Scythe

	Preimmune IP GST release	Preimmune IP Reaper release
Minutes:	50 100 150 200 250	50 100 150 200 250

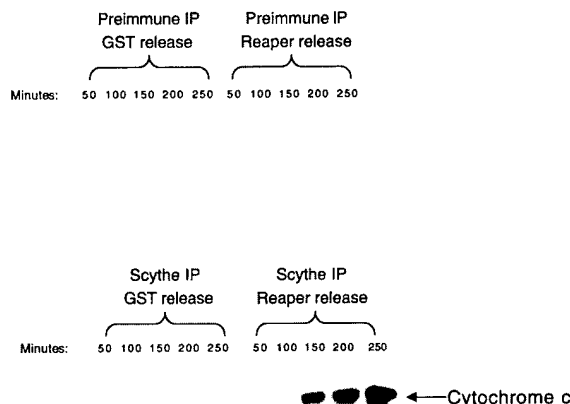|	Scythe IP GST release	Scythe IP Reaper release
Minutes:	50 100 150 200 250	50 100 150 200 250

◀—Cytochrome c

Fig. 5. The pro-apoptotic activity released from Scythe upon Reaper treatment induces direct release of cytochrome *c* from isolated mitochondria. Mitochondria isolated from *Xenopus* egg extracts and further purified by centrifugation through a percoll gradient were diluted 1:10 into ELB containing an ATP-regeneration mix. The indicated supernatants obtained as described above were added 1:10 to the mitochondria at room temperature and at the indicated times 25 μl of the mixture were filtered through a 0.1 μM microfilter. Aliquots of filtrate (10 μl) were separated by SDS–PAGE and processed for Western blotting using an anti-cytochrome *c* monoclonal antibody.

by Reaper, we added recombinant Bcl-xL protein to the released supernatant; this protein very effectively inhibited the induction of caspase activity by the released factor(s) (Figure 4C).

The pro-apoptotic activity released from Scythe is a direct inducer of mitochondrial cytochrome c release

As we reported previously, neither the C312 variant of Scythe nor recombinant Reaper can induce cytochrome *c* release from purified mitochondria in the absence of additional cytosolic components (Thress *et al.*, 1998). Reaper added along with full-length recombinant Scythe is also inactive in this assay, reinforcing the notion that accessory factors are required for Scythe/Reaper-induced cytochrome *c* release. Potentially, the material released from Scythe immunoprecipitates by Reaper addition might contain such factors. We incubated the various released supernatants described above with purified mitochondria. As shown in Figure 5, the supernatant obtained from the Reaper-treated Scythe immunoprecipitates triggered direct cytochrome *c* release from isolated mitochondria, while only low levels of background cytochrome *c* efflux were observed in mitochondria treated with control supernatants or incubated with buffer alone. These data indicate that Scythe sequesters cytochrome *c*-releasing activity which is liberated following binding of Scythe to Reaper.

Scythe is not required downstream of mitochondrial cytochrome c release

Although the experiments described above firmly place Scythe upstream of mitochondrial cytochrome *c* release in the pathway of Reaper-induced apoptosis, they do not preclude the possibility that Scythe plays an additional post-cytochrome *c* role. To address this, we immuno-depleted Scythe from the egg extract and asked whether addition of pure cytochrome *c* to the depleted extract could still induce caspase activation and morphological
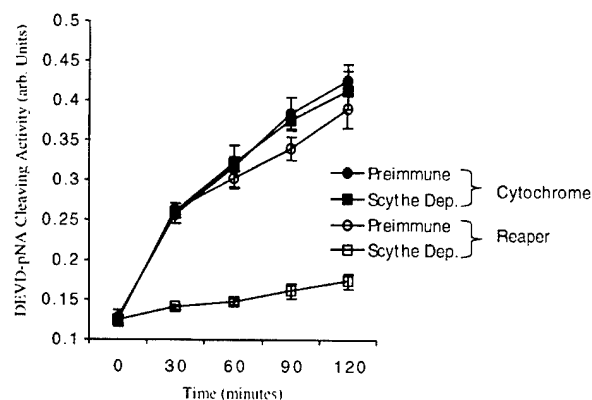
Fig. 6. Scythe acts exclusively upstream of mitochondria. Recombinant Reaper protein (300 ng/μl) or equine heart cytochrome *c* (1 ng/μl) were added to either *Xenopus* egg extract depleted of endogenous Scythe protein (Scythe Dep.) or extracts similarly treated with pre-immune sera (Preimmune). At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity.

apoptosis. As shown in Figure 6, Scythe depletion was unable to interfere, even partially, with cytochrome *c*-induced caspase activation. Thus, Scythe is not required after efflux of cytochrome *c* from the mitochondria.

Excess IAPs neither prevent Reaper-induced cytochrome c release nor disrupt the Reaper–Scythe interaction

It has been reported by several groups that overexpression of IAPs can block Reaper-induced apoptosis (Hay *et al.*, 1995; Vucic *et al.*, 1997a; McCarthy and Dixit, 1998). Moreover, IAPs can bind directly not only to Reaper, but to other critical regulators of *Drosophila* apoptosis: Grim and Hid (Kaiser *et al.*, 1998; Vucic *et al.*, 1998). While IAPs reportedly inhibit caspase activity and pro-caspase activation, their ability to inhibit upstream events, such as mitochondrial cytochrome *c* release, has not been examined (Roy *et al.*, 1997; Deveraux *et al.*, 1998). To test this, we produced the three BIR domains of c-IAP 1, previously reported to be an effective inhibitor of Reaper-induced apoptosis, in bacteria (McCarthy and Dixit, 1998). After purifying the BIR protein, we added it to egg extracts together with Reaper. At concentrations of BIR that very effectively blocked Reaper-induced apoptosis, we observed no inhibition of Reaper-induced cytochrome *c* release (Figure 7A and B). Even when present at a 10-fold molar excess to Reaper, this protein blocked neither Reaper-induced cytochrome *c* release nor binding of Reaper to Scythe (Figure 7C).

Since the first 15 amino acids of Reaper are critical for IAP binding (McCarthy and Dixit, 1998) and excess IAP protein did not interfere with the Scythe–Reaper interaction, we assumed that IAPs and Scythe protein must interact with Reaper at distinct sites. In accordance with this, we found that full-length Reaper protein and a mutant Reaper protein lacking the first 15 amino acids (Rpr 16–65) were both capable of binding Scythe (Figure 8A). Moreover, a fusion protein consisting of GST linked to the first 15 amino acids of Reaper was unable to bind to Scythe (Figure 8A). As has been reported in other systems using similar N-terminal Reaper mutants, Rpr 16–65 could still trigger caspase activation and morphological apoptosis upon addition to *Xenopus* egg extracts, but less efficiently
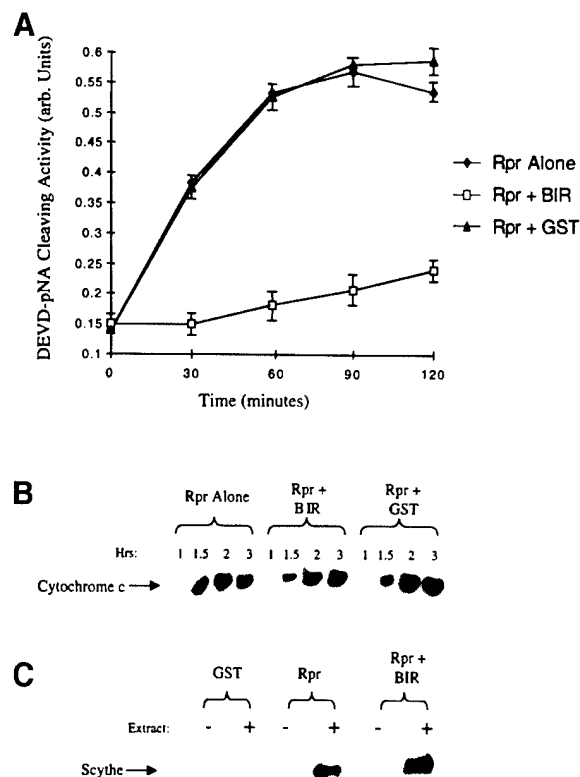

Fig. 7. Excess BIR protein inhibits Rpr-induced apoptosis but not Rpr-induced cytochrome *c* release. (**A**) Recombinant Reaper (Rpr) protein alone (300 ng/μl) or Reaper in combination with equivalent amounts of either recombinant BIR or GST proteins were added to crude *Xenopus* egg extracts. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (**B**) Samples were processed as in (A), but 15 μl aliquots were filtered through a 0.1 μM microfilter and processed for immunoblotting with an anti-cytochrome *c* monoclonal antibody. (**C**) Recombinant GST, GST–Reaper and GST–Reaper pre-incubated with a 10-fold molar excess of recombinant BIR protein were immobilized on glutathione–Sepharose beads and incubated in the absence (–) or presence (+) of *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 16 amino acids of the *Xenopus* Scythe protein.

than the similarly added wild-type Reaper protein (data not shown) (Chen *et al.*, 1996a; Vucic *et al.*, 1997b).

The Reaper, Grim and Hid proteins are not notably homologous outside of a region of limited homology found at their extreme N termini (corresponding to the first 15 amino acids of Reaper), which appears to be responsible for their shared ability to bind IAPs (Chen *et al.*, 1996b; McCarthy and Dixit, 1998). Surprisingly, despite the fact that Scythe could bind to a region of Reaper with no overt primary sequence homology to Grim and Hid proteins, GST–Hid and GST–Grim proteins bound Scythe nearly as well as did GST–Reaper (Figure 8B). Several control proteins tested, including GST alone, did not bind to Scythe. Therefore, although the primary sequences of these proteins do not reveal an obvious shared motif, Grim, Hid and Reaper can all interact with Scythe.

Discussion

Reaper protein has no evident catalytic activity and only limited homology to other apoptotic regulators, yet it is a

A

GST   Rpr F.L.   Rpr 16-65   Rpr 1-15

Extract: − +   − +   − +   − +

Scythe ⟶

B

GST   Rpr F.L.   Grim   Hid

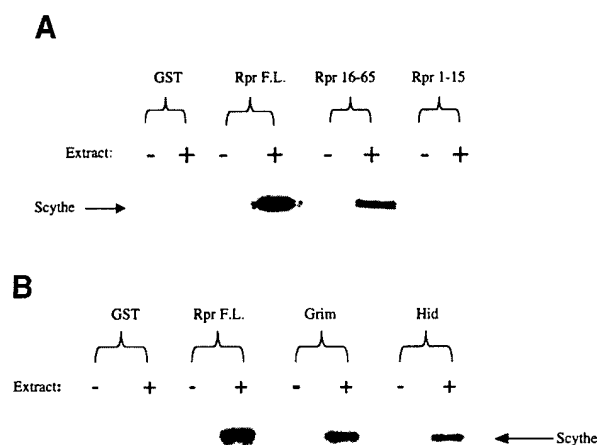Extract: − +   − +   − +   − +

◄⸺ Scythe

Fig. 8. Scythe interacts with the C-terminal 50 amino acids of Reaper as well as with the *Drosophila* apoptotic regulators Hid and Grim. (A) Recombinant GST protein or various GST–Reaper fusion proteins were immobilized on glutathione–Sepharose beads and incubated in the absence (−) or presence (+) of *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 16 amino acids of the *Xenopus* Scythe protein. Rpr F.L., full-length Reaper (amino acids 1–65); Rpr 16–65, amino acids 16–65 of Reaper; Rpr 1–15, amino acids 1–15 of Reaper. (B) Recombinant GST protein alone or the indicated GST fusion proteins were immobilized on glutathione–Sepharose beads, incubated in the absence (−) or presence (+) of *Xenopus* egg extract for 1 h at 4°C and treated as described in (A).

potent inducer of apoptosis in cells of both lepidopteran and vertebrate origin. In this report, we demonstrate that Reaper acts, at least in part, by inducing the dissociation of a Scythe-bound factor that can trigger direct release of mitochondrial cytochrome *c*.

Scythe sequesters a positive apoptotic regulator

Neither C312 Scythe, Reaper alone nor Reaper added with full-length Scythe can induce direct mitochondrial cytochrome *c* release; the experiments presented here make a strong case that the cytochrome *c*-releasing factor(s) which operates downstream of Reaper is initially bound to Scythe and maintained in an inactive form. While it is not yet clear how Reaper induces the dissociation of these factors from Scythe, it is attractive to speculate that Reaper binding induces a conformational change in Scythe leading to desequestration of the bound factor(s). Alternatively, it is possible that Reaper displaces the factor competitively through binding to the same site on Scythe.

Although we have not yet identified the Scythe-bound factor(s) responsible for the cytochrome *c*-releasing activity, several Bcl-2 family members have been implicated in the direct release of mitochondrial cytochrome *c* (Li *et al.*, 1998; Luo *et al.*, 1998; Desagher *et al.*, 1999). Thus, we consider it quite possible that a Bcl-2 family member is one of the Scythe-bound factors. Although the cytochrome *c*-releasing activity of pro-apoptotic Bcl-2 family members may be activated by caspase cleavage [e.g. bid cleavage by caspase 8 (Li *et al.*, 1998; Luo *et al.*, 1998)], the Scythe-sequestered factor probably does not require caspases for activity because caspase inhibitors do not appear to prevent Reaper-induced cytochrome *c* release (Evans *et al.*, 1997a). While the ability of Bcl-xL protein

to inhibit the activity of factor 'X' is consistent with the hypothesis that a positively acting Bcl-2 family member may be sequestered by Scythe, attempts to test this hypothesis by immunoblotting of anti-*Xenopus* Scythe immunoprecipitates with anti-Bcl-2 family sera have been hampered by the lack of cross-reactivity of the available antisera with homologous *Xenopus* proteins. However, preparation of anti-human Scythe antisera should soon facilitate the examination of factors associated with human Scythe. Four specific Scythe-bound proteins can be detected in *Xenopus* anti-Scythe immunoprecipitates by silver staining of SDS–PAGE gels, but their identity remains to be determined (K.Thress and S.Kornbluth, unpublished).

An alternative hypothesis of Scythe function to be considered is that Scythe may be part of an Apaf-1-like complex, which, upon binding Reaper, promotes the processing of a pro-caspase that acts upstream of mitochondrial cytochrome *c* release. High levels of broad-spectrum caspase inhibitors do not appear to prevent Reaper-induced cytochrome *c* release, but this does not rule out the involvement of a caspase insensitive to the inhibitors used in those experiments (Evans *et al.*, 1997a). Since exogenous Scythe can resequester the pro-apoptotic factor(s) released by Scythe, an Apaf-1/pro-caspase-like model for Scythe function would have to postulate that Scythe can re-bind and neutralize the released and activated caspase.

The C312 Scythe protein probably acts as a dominant-negative Scythe variant

Data presented in Figure 4B illustrate that the Scythe C312 protein, which can induce apoptosis independently of Reaper, cannot, like full-length Scythe, suppress the activity of pro-apoptotic factors released from Scythe. However, as reported previously, a resin linked to C312 Scythe very effectively depletes *Xenopus* egg extracts of factors required for Reaper-induced cytochrome *c* release and caspase activation (Thress *et al.*, 1998). It is possible that the C312 protein assumes an 'active' conformation that triggers activation of a bound cytochrome *c*-releasing factor. However, we have found that the amount of C312 protein required to induce apoptosis in egg extracts exceeds the level of endogenous Scythe by at least 2-fold (data not shown). Collectively, these data suggest that the C312 protein may act, not as an activated variant of Scythe, but as a dominant interfering Scythe protein. Possibly, both full-length and C312 Scythe can bind to pro-apoptotic factors, but only the full-length Scythe can inhibit their activity.

Two pathways of Reaper-induced apoptosis?

Since excess IAP protein did not appear to block Reaper-induced mitochondrial cytochrome *c* release, while effectively blocking Reaper-induced apoptosis, it is entirely possible that excess IAPs prevent Reaper-induced apoptosis primarily through post-mitochondrial inhibition of pro-caspase activation. Indeed, purified IAP protein very effectively prevents activation of pro-caspases 9 and 3 upon addition of purified cytochrome *c* to the *Xenopus* egg extract (K.Thress and S.Kornbluth, unpublished). What then is the role of Reaper binding to IAPs? The 16–65 Reaper variant is less active than the wild-type

Reaper protein, suggesting that the first 15 amino acids of Reaper may serve a pro-apoptotic function. This is the region of Reaper that also binds IAPs, prompting the speculation that Reaper binding may serve to inactivate an anti-apoptotic function of IAPs in the egg extract, rather than IAPs acting to incapacitate Reaper. Indeed, it has recently been demonstrated that Reaper, Grim and Hid proteins can block the ability of a *Drosophila* IAP to suppress caspase-dependent death of yeast. Moreover, it was shown that the N-terminal region of Hid, which is homologous to Reaper, mediated its IAP-suppressing activity (Wang *et al.*, 1999).

Because the 16–65 protein retains the ability to interact with Scythe, it is likely that Scythe mediates the residual apoptosis-inducing activity of the 16–65 Reaper protein. Interestingly, when this protein is added to egg extracts at 4- to 5-fold higher levels than wild-type Reaper protein, the 16–65 and wild-type proteins induce roughly equivalent levels of caspase activity (data not shown). This suggests that Scythe-dependent pathways, when sufficiently activated, may be able to compensate for the absence of pathways (possibly IAP inhibition) which normally act coordinately with Scythe to mediate Reaper-induced apoptosis.

Scythe in vertebrates

The conservation of Scythe protein across species, coupled with the ability of *Drosophila* Reaper to trigger the dissociation of cytochrome *c*-releasing factors from *Xenopus* Scythe, argues strongly that a similarly acting Scythe ligand must exist in vertebrates. Reaper, Grim and Hid proteins have all been shown to induce apoptosis in human cells (Claveria *et al.*, 1998; McCarthy and Dixit, 1998; Haining *et al.*, 1999). These proteins share the ability to bind IAPs and, as demonstrated here, have the common ability to bind Scythe. We have also found that these proteins can bind to an *in vitro* translated form of the human Scythe protein (data not shown). Whether there will be several distinct Scythe ligands that share primary sequence homology to Grim, Hid or Reaper proteins remains to be determined. It will also be of great interest to determine whether regulated release of Scythe-sequestered cytochrome *c*-releasing factors is important for other, non-Reaper-mediated, pathways of apoptosis.

Materials and methods

Preparation of GST fusion proteins

Two separate truncations of recombinant *Drosophila* Reaper protein were constructed: the N-terminal 15 amino acids (Rpr 1–15) and the C-terminal 50 amino acids (Rpr 16–65). cDNAs encoding these truncations were PCR amplified using the following primers. Rpr 1–15: 5'-GATCGGATCCATGGCAGTGGCATTC-3'; 5'-GATCAAGCTTTC-ACCGCAACAGAGTCGC-3'. Rpr 16–65: 5'-GATCCCATGGAGG-CGGAGCAGAAGGAGCAG-3'; 5'-GATCAAGCTTTCATTGCGATG-GCTTGCGATA-3'. Full-length *Drosophila* Grim and HID were also PCR amplified using the following primers. Grim: 5'-GATC-GGATCCATGGCCATCGCCTATTTC-3'; 5'-GATCAAGCTTTTAGTT-CTCCTTGGAGGTGGCATC-3'. HID: 5'-GATCGGATCCATGGCCG-TGCCCTTTTATTTG-3'; 5'-GATCAAGCTTTCATCGCGCCGCAAA-GAAGCC-3'. cDNA encoding a truncated hIAP-1 protein consisting of the three BIR domains, but lacking the C-terminal RING finger domain, was amplified using the following primers: 5'-GATCGGAT-TCATGAACATAGTAGAAAAAC-3'; 5'-GATCAAGCTTTCATGTTCT-TTCTTCTGGTAG-3'. PCR fragments were cloned into the expression vector Gex KG, a derivative of Gex 2T (Pharmacia) containing additional

polylinker sites and a polyglycine insert, and transformed into the Topp 1 bacterial strain (Stratagene). Recombinant protein was produced as described previously (Evans *et al.*, 1997a). Control GST protein was expressed and prepared in a manner identical to that used for all other GST fusion proteins.

Preparation of Xenopus egg extracts

For induction of egg laying, mature female frogs were injected with 100 U of pregnant mare serum gonadotropin (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (HCG; USB). Fourteen to 20 h after injection with HCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine (pH 7.8), washed three times in modified Ringer's solution (MMR) (1 M NaCl, 20 mM KCl, 10 mM MgSO$_4$, 25 mM CaCl$_2$, 5 mM HEPES pH 7.8, 0.8 mM EDTA), and then washed in egg lysis buffer [ELB: 250 mM sucrose, 2.5 mM MgCl$_2$, 1.0 mM dithiothreitol (DTT), 50 mM KCl, 10 mM HEPES] pH 7.4. Eggs were packed by low-speed centrifugation at 400 *g*. Following the addition of aprotinin and leupeptin (final concentration 5 mg/ml), cytochalasin B (final concentration 5 mg/ml) and cycloheximide (final concentration 50 mg/ml), eggs were lysed by centrifugation at 10 000 *g* for 15 min. For nuclear formation, extracts were supplemented with demembranated sperm chromatin (1000 nuclei/µl) and an ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP and 50 mg/ml creatine phosphokinase). Recombinant proteins added to extracts were diluted in XB buffer (50 mM sucrose, 100 mM KCl, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM K–HEPES, pH 7.7) and added at a concentration of 300 ng/µl, unless indicated otherwise.

Immunodepletion assays

Protein A–Sepharose beads were washed in ELB and pre-incubated with 10 mg/ml bovine serum albumin in ELB for 40 min at 4°C. The beads were washed twice more with ELB and 10 µl of Sepharose beads were incubated with 100 µl of pre-immune or anti-Scythe antisera at 4°C for 70 min. The beads were washed again with ELB and then incubated with 100 µl of the crude *Xenopus* egg extract. After 1 h at 4°C, the antibody–bead complexes were pelleted, the supernatant was transferred to a fresh microfuge tube and the depletion process was repeated, using fresh beads, twice more. This depleted extract was then assayed for the ability to induce apoptotic nuclear fragmentation, cytochrome *c* release and/or caspase activity directed against artificial substrate (DEVDase) activation.

DEVDase assays

To measure caspase activity, 3 µl of each sample were incubated with 90 µl of assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric substrate *N*-acetyl-DEVD-*p*-nitroanilide (Ac-DEVD-pNA) (final concentration 200 mM: Biomol Caspase-3 assay system) at 37°C. Absorbance was measured at 405 nm at various time points in a LabSystems MultiSkan MS microtiter plate reader. All measurements were repeated in triplicate for each time point and the average was reported.

Scythe release assays

Either anti-Scythe or pre-immune sera linked to protein A–Sepharose beads were used to immunoprecipitate from *Xenopus* egg extract as described above. Following three successive rounds of precipitation, the beads were combined and washed three times in ELB. The beads were resuspended in ELB and incubated with recombinant, soluble GST or GST–Reaper protein (added 1:10, protein:bead volume) for 30 min at room temperature. The beads were then pelleted by centrifugation and the supernatant concentrated in microcon 10s (Amicon) by centrifugation for 20 min at 4°C. Following concentration, the supernatant was added 1:10 to extracts depleted of endogenous Scythe protein, the extracts were incubated at room temperature, and at the indicated times 3 µl aliquots were collected for DEVD-pNA cleavage activity.

Mitochondrial cytrochrome c release assays

To fractionate the crude egg extract into cytosolic and membranous components, the crude extract was centrifuged further at 55 000 r.p.m. (200 000 *g*) in a Beckman TLS-55 rotor for the TL-100 centrifuge for 1 h. The heavy membrane fraction (enriched in mitochondria) was removed and the mitochondrial fraction was purified further by centrifugation of the heavy membrane through a percoll gradient consisting of 42, 37, 30 and 25% percoll in mitochondria isolation buffer (1 M sucrose, 100 mM ADP, 2.5 M KCl, 1 M DTT, 1 M succinate, 1 M HEPES–KOH pH 7.5, 0.5 M EGTA, 1.5 M mannitol) for 25 min at

25 000 r.p.m. with no brake in the TLS-55 rotor. The isolated heavy membrane fraction containing mitochondria was diluted 1:10 into ELB containing an ATP-regenerating cocktail (10 mM phosphocreatine, 2 mM ATP and 50 mg/ml creatine phosphokinase). At various time points, cytochrome c content was analyzed after filtering 25 μl of the mixture through a 0.1 μm ultrafree-MC filter (Millipore). Aliquots of 10 μl protein were then separated by SDS–PAGE and immunoblotted with an anti-cytochrome c monoclonal antibody (Pharmingen), horseradish peroxidase-linked anti-mouse sera and an ECL chemiluminescence detection system (Amersham).

Acknowledgements

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Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper

Kenneth Thress, Jaewhan Song[1], Richard I. Morimoto[1] and Sally Kornbluth[2]

Department of Pharmacology and Cancer Biology, C370 LSRC, Research Drive, Duke University Medical Center, Durham, NC 27710 and [1]Rice Institute for Biomedical Research, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 North Campus Drive, Evanston, IL 60208, USA

[2]Corresponding author

Protein folding mediated by the Hsp70 family of molecular chaperones requires both ATP and the co-chaperone Hdj-1. BAG-1 was recently identified as a bcl-2-interacting, anti-apoptotic protein that binds to the ATPase domain of Hsp70 and prevents the release of the substrate. While this suggested that cells had the potential to modulate Hsp70-mediated protein folding, physiological regulators of BAG-1 have yet to be identified. We report here that the apoptotic regulator Scythe, originally isolated through binding to the potent apoptotic inducer Reaper, shares limited sequence identity with BAG-1 and inhibits Hsp70-mediated protein refolding. Scythe-mediated inhibition of Hsp70 is reversed by Reaper, providing evidence for the regulated reversible inhibition of chaperone activity. As Scythe functions downstream of Reaper in apoptotic induction, these findings suggest that Scythe/Reaper may signal apoptosis, in part through regulating the folding and activity of apoptotic signaling molecules.
Keywords: Hsp70 inhibition/Scythe/Reaper

Introduction

The Hsp70 chaperone proteins facilitate proper protein folding, prevent protein aggregation and assist in the assembly of multi-protein complexes. In this way, Hsp70 family members monitor and counteract the accumulation of potentially harmful misfolded polypeptides, particularly following exposure of the cell to stressful conditions (Hartl, 1996). Comprised of both constitutive and induced members, Hsp70 family members share the ability to recognize exposed hydrophobic patches on non-native proteins and promote their re-folding (Rassow et al., 1995; Rudiger et al., 1997). However, this protein folding requires, in addition to Hsp70/Hsc70, both ATP and a 'co-chaperone'. The most intensively studied of these co-chaperones, Hdj-1, enhances ATP hydrolysis and concomitant release of the folded protein substrate (Hohfeld et al., 1995; Minami et al., 1996).

In vitro, purified Hsc70 releases non-native substrates in the presence of ATP. However, it was recently reported that Hsp70 can associate *in vivo* with BAG-1, a protein that prevents release of folded protein substrates, even in the presence of Hdj-1 and ATP (Hohfeld and Jentsch, 1997; Takayama et al., 1997; Demand et al., 1998; Stuart et al., 1998; Nollen et al., 2000). Indeed, BAG-1, the first reported negative regulator of Hsp70 function, forms ternary complexes with Hsp70, Hdj-1 and the substrate, maintaining the substrate in a partially folded, yet soluble state (Bimston et al., 1998; Luders et al., 2000a,b). In effect, without inhibiting Hsp70-mediated nucleotide hydrolysis, BAG-1 uncouples ATP hydrolysis from release of the folded substrate (Bimston et al., 1998).

Although its biochemical role in modulating Hsp70 function is clear, the precise biological function of BAG-1 is not known. Originally isolated as a bcl-2-interacting protein, BAG-1 was subsequently shown to associate with other signaling molecules, including Raf-1, the intracellular domain of the PDGF receptor and a number of steroid hormone receptors (Takayama et al., 1995; Zeiner and Gehring, 1995; Bardelli et al., 1996; Wang et al., 1996; Song et al., 2001). Intriguingly, under some circumstances, overexpression of BAG-1 was reported to have anti-apoptotic activity (Takayama et al., 1995). Structurally, BAG-1 does not have any particularly striking features; however, as originally described by Reed and colleagues, BAG family proteins do share a conserved C-terminal ~50 amino acid motif dubbed the 'BAG' domain (Takayama et al., 1999). In addition, BAG-1 and several of its relatives have an N-terminal domain with a high degree of homology to ubiquitin. The functional significance of this homology is not yet clear.

We recently isolated an apoptotic regulator called Scythe, whose N-terminus, like that of BAG-1, bears marked homology to ubiquitin (Thress et al., 1998). Scythe acts downstream of Reaper, a small (65 amino acid) protein that was identified in a screen for apoptotic regulators in the fly, *Drosophila melanogaster*. Genetic evidence has implicated Reaper as an important mediator of apoptosis both during development and following DNA damage (White et al., 1994, 1996). Although Reaper homologs have not yet been identified in other systems, fly Reaper can induce apoptosis in human cells and can trigger biochemical hallmarks of apoptosis (mitochondrial cytochrome *c* release, caspase activation) in cell-free extracts prepared from *Xenopus* (Evans et al., 1997; McCarthy and Dixit, 1998). These data suggest that Reaper-responsive pathways are highly conserved.

Using recombinant Reaper as an affinity resin, Scythe was purified as a high-affinity Reaper interactor (Thress et al., 1998). As immunodepletion of Scythe from *Xenopus* egg cell-free extracts prevented both Reaper-induced cytochrome *c* release and caspase activation, it appeared that Scythe acted downstream of Reaper in the pathway of apoptotic induction. Further studies revealed that Scythe was actually a negative regulator of apoptosis, acting to
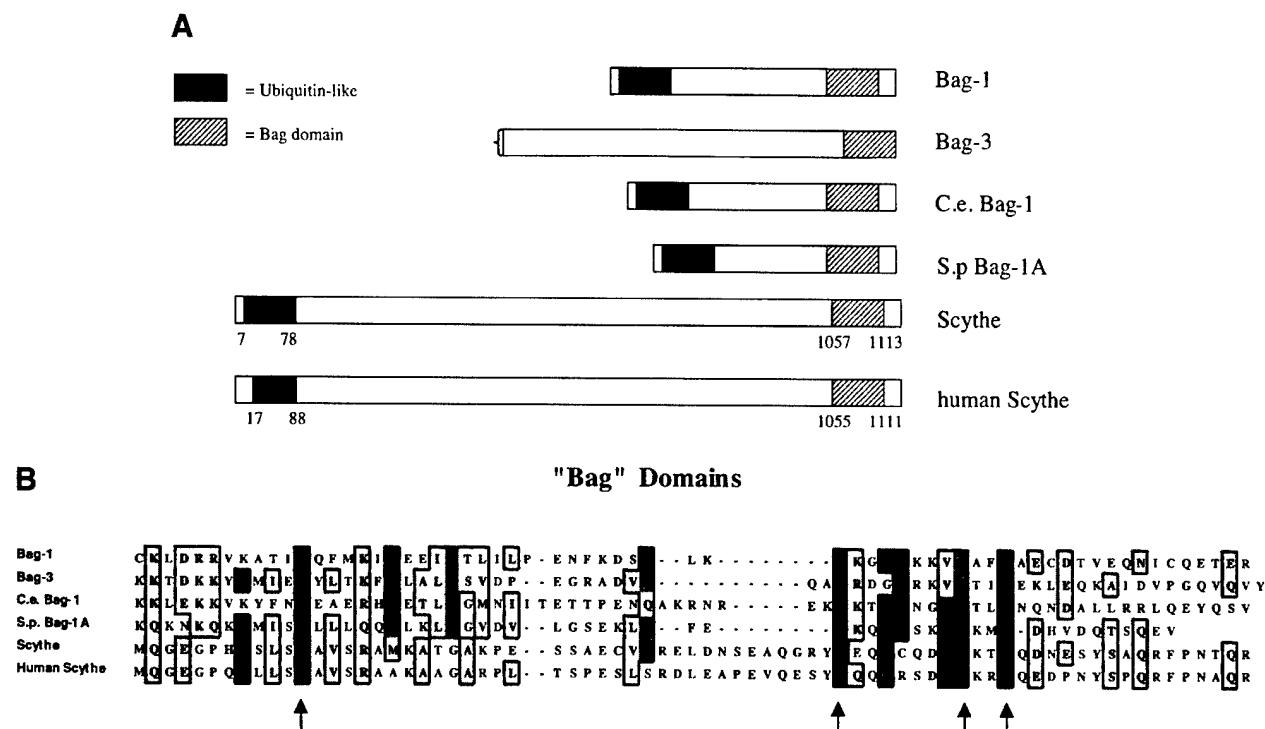
Fig. 1. Scythe structurally resembles BAG family proteins. (A) The domains of several BAG family members along with *Xenopus* and human Scythe showing the relative positions of the ubiquitin-like motif (black) and C-terminal 'BAG' domain (striped). The complete open reading frame of BAG-3 has yet to be fully sequenced. C.e., *Caenorhabditis elegans*; S.p., *Schizosaccharomyces pombe*. (B) Alignment of the C-terminal BAG domains of the proteins in (A). Dark gray and light gray shading indicate identical and similar residues, respectively.

sequester an as yet unidentified direct inducer of mitochondrial cytochrome *c* release (Thress *et al.*, 1999). Upon binding of Reaper, Scythe released this factor(s), leading to mitochondrial cytochrome *c* release, caspase activation and full apoptosis. This series of events was recapitulated in a semi-purified system in that immuno-precipitates of Scythe, when washed extensively and incubated with Reaper, released a factor(s) capable of initiating cytochrome *c* release directly from purified mitochondria.

In experiments reported here, we show that the apparent similarities between BAG-1 and Scythe (the presence of an N-terminal ubiquitin domain, anti-apoptotic activity) are likely to be more than superficial. Indeed, we show that Scythe, like BAG-1, is a direct inhibitor of Hsp70 protein folding activity. Moreover, a BAG domain in Scythe mediates this inhibition. However, while the physiological means of reversing BAG-1-mediated Hsp70 inhibition are not known, we have found that Reaper can relieve Scythe-mediated repression of Hsp70. These data provide the first evidence for reversibility of Hsp70 inhibition by a co-chaperone ligand.

Results

Scythe bears structural similarity to BAG family proteins

As both Scythe and BAG-1 are anti-apoptotic when overexpressed, and share, along with other BAG family proteins, an N-terminal ubiquitin-like domain, we were interested in the possibility that Scythe might also contain a BAG domain. Clustal alignments of BAG family members and both human and *Xenopus* Scythe proteins

revealed candidate C-terminal BAG domains present in Scythe molecules from both species (Figure 1). While the overall similarity of the BAG domain across different proteins is ~30%, four strictly conserved residues, found in all BAG family members, are also conserved in Scythe.

Scythe binds to the ATPase domain of Hsc70/ Hsp70 in a BAG domain-dependent fashion

As described above, BAG-1 protein is unusual in its ability to inhibit the protein folding activity of Hsc70/Hsp70 family members; this inhibitory activity depends upon the presence of the BAG domain, which provides a direct binding site for Hsc70/Hsp70 proteins. The presence of a putative BAG domain on Scythe prompted us to examine whether it too might bind Hsc70/Hsp70 proteins. *Xenopus* egg extract was supplemented with radiolabeled, *in vitro* translated Hsc70 protein and incubated with Sepharose beads linked to either glutathione *S*-transferase (GST) or GST fused to the C-terminal half of Scythe (Scythe C312). After pelleting and extensive washing, these resins were examined by SDS–PAGE for the presence of bound Hsc70. As shown in Figure 2A, Hsc70 bound specifically to the Scythe resin. Similarly, Sepharose-linked GST–Hsc70 efficiently bound endogenous *Xenopus* Scythe from egg extracts, at levels comparable to those obtained using GST–Reaper as bait (Figure 2B). To demonstrate that endogenous Scythe and Hsc70 proteins were able to interact, we immunoprecipitated Hsc70 from *Xenopus* egg extracts and immunoblotted the samples with anti-Scythe sera. As shown in Figure 2C, endogenous Hsc70 and Scythe proteins co-immunoprecipitated, while Scythe did not associate with a control antibody (anti-Wee1) or protein A–Sepharose alone.

To determine whether Scythe–Hsc70 interactions could be observed between human proteins in intact cells, full-length myc-tagged human Scythe transfected into 293T cells was immunoprecipitated from cell lysates with anti-myc antibody and examined for the presence of bound Hsc70 protein. As anticipated, human Scythe and endogenous Hsc70 could, like their *Xenopus* counterparts, be co-precipitated. Importantly, as reported for BAG-1 protein, deletion of the BAG domain (hScythe ΔC; removal of the C-terminal 81 amino acids of Scythe), but not the N-terminal ubiquitin domain (hScythe ΔN) from Scythe completely abrogated binding to Hsc70 (Figure 2D), despite equal levels of expression of mutant and wild-type proteins (Figure 2E). Collectively, these data indicate that Scythe binds specifically to Hsc70/Hsp70 and that this binding is mediated by Scythe's C-terminal BAG domain.

As Scythe, like BAG-1 protein, associates with Hsc70 through its BAG domain, we wished to determine whether Scythe also behaved like BAG-1 in its ability to interact specifically with the ATPase domain of Hsc70/Hsp70. Accordingly, we conducted a series of binding studies using His-tagged Scythe produced in baculovirus vectors and bacterially produced GST-tagged Hsp70 proteins. As was seen in the 293T lysates, full-length Hsp70 bound efficiently to full-length Scythe, while Scythe ΔC was greatly impaired in its ability to bind Hsp70 (Figure 2F). Importantly, full-length Scythe was also able to bind to the isolated ATPase domain of Hsp70, while deletion of the BAG domain from Scythe completely abrogated this association. These data are consistent with previously reported findings demonstrating that human Scythe can bind to a short sequence within the ATPase domain of the Hsp70-like protein, Stch (Kaye *et al.*, 2000).

Scythe functions as a negative regulator of Hsp70 chaperone activity

Among the group of co-chaperones/Hsp70 interactors, BAG-1 is the only protein reported to negatively regulate the protein folding ability of Hsc70/Hsp70 proteins (Takayama *et al.*, 1997; Bimston *et al.*, 1998; Nollen *et al.*, 2000). Given the parallels between Scythe and BAG-1, we wished to determine whether Scythe might be a functional relative of BAG-1, able to negatively modulate the protein folding ability of Hsp70. Typically, *in vitro* protein folding assays examine the ability of Hsp70 chaperones to refold denatured test substrates. These assays appear to reflect faithfully the modulation of Hsp70 activity by both activators and inhibitors, even though the test substrates are not the true *in vivo* targets of
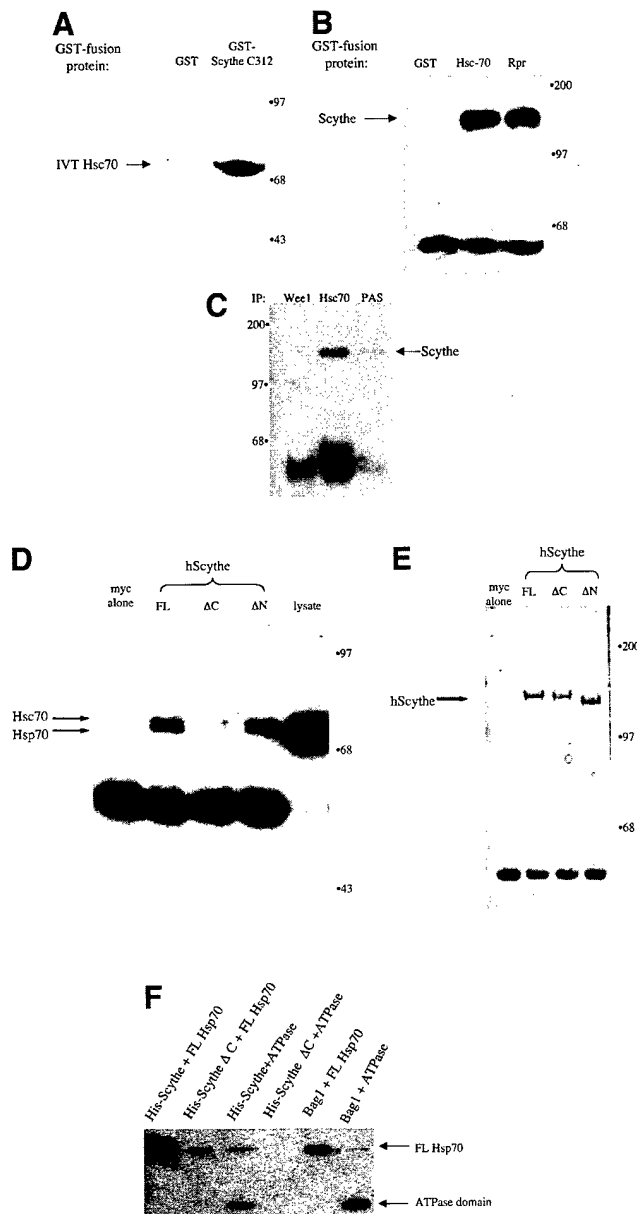

Fig. 2. Scythe binds Hsp70/Hsc70 in a BAG domain-dependent fashion. (**A**) *In vitro* translated *Xenopus* Hsc70 (IVT Hsc70) was added to extracts and incubated for 30 min at 4°C. GST or GST–Scythe C312 immobilized on glutathione–Sepharose beads was added to the extract and incubated for an additional 30 min. The beads were then washed three times with egg lysis buffer (ELB), resolved by SDS–PAGE and bands were visualized by autoradiography. (**B**) GST or the indicated GST fusion protein was immobilized on glutathione–Sepharose beads and incubated in the presence of *Xenopus* egg extract for 1 h at 4°C. The beads were then washed three times with ELB, resolved by SDS–PAGE and processed for western blotting with an anti-Scythe polyclonal antibody. (**C**) Antibodies against *Xenopus* Wee1 or Hsc70 were coupled to Protein A–Sepharose (PAS) beads and then incubated in *Xenopus* egg extract for 1 h at 4°C. Immunoprecipitates were washed three times with ELB, resolved by SDS–PAGE and processed for immunoblotting with an anti-Scythe polyclonal antibody. (**D**) 293T cells were transfected with 3 µg of either the indicated myc-tagged human Scythe construct (hScythe) or the parental myc plasmid alone (myc alone). Thirty-six hours after transfection, cells were lysed, centrifuged, and the supernatants incubated with a monoclonal myc antibody for 1 h at 4°C. PAS beads were then added and, after an additional 1 h incubation, the beads were pelleted, washed three times in lysis buffer, and bound proteins were resolved by SDS–PAGE. After western transfer, the blots were probed with an anti-Hsp70/Hsc70 monoclonal antibody. (**E**) The identical samples processed in (D) were run on a parallel SDS gel and proteins were stained with Coomassie Brilliant Blue. (**F**) His-tagged Scythe, His-tagged Scythe lacking the BAG domain (His-Scythe ΔC) or His-tagged BAG-1 was incubated in the presence of either full-length Hsp70 (FL Hsp70) or the ATPase domain of Hsp70 (ATPase) for 1 h at 4°C. His-tagged proteins were recovered using a nickel resin and bound proteins were separated by SDS–PAGE. After western transfer, the blots were probed with an anti-Hsp70 monoclonal antibody.
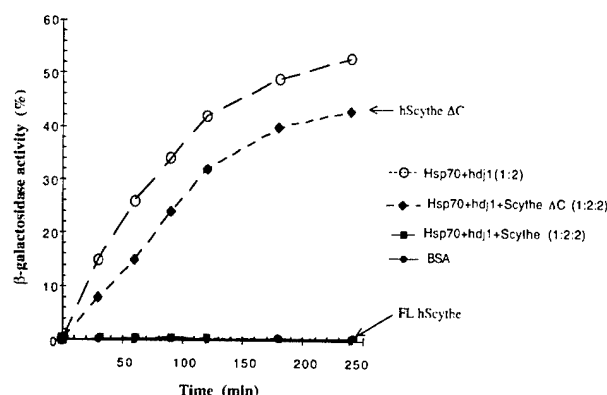
Fig. 3. Scythe functions as a negative regulator of Hsp70 chaperone activity. The inhibitory effect of full-length human Scythe (FL hScythe, 3.2 µM) and a truncated Scythe mutant lacking the 'BAG' domain (Scythe ΔC, 3.2 µM) on Hsp70-dependent refolding was examined by the percentage recovered activity of unfolded β-galactosidase (3.2 nM) diluted into a refolding buffer containing ATP (1 mM), Hsp70 (1.6 µM) and Hdj-1 (3.2 µM). As a control for spontaneous refolding, denatured β-galactosidase (3.2 nM) was diluted into refolding buffer containing 3.2 µM bovine serum albumin (BSA).

the modulator–Hsp70 complex (Takayama *et al.*, 1997; Nollen *et al.*, 2000). Accordingly, we asked whether Scythe could alter the ability of Hsp70 to refold denatured β-galactosidase. Following denaturation in 6 M guanidine HCl, recombinant β-galactosidase was incubated in refolding buffer with purified recombinant Hsp70, ATP and the co-chaperone Hdj-1 in the presence or absence of baculovirus-produced Scythe protein. After various times of incubation, samples were assayed for β-galactosidase activity using the colorimetric substrate *o*-nitrophenyl-β-D-galactopyranoside. As reported previously, recombinant Hsp70/Hdj-1 allowed refolding of denatured β-galactosidase, with 50% recovery of enzymatic activity (Figure 3). However, when full-length Scythe was added to the assay at a 1:2 molar ratio with Hsp70, the Hsp70-mediated refolding of denatured β-galactosidase was completely inhibited (Figure 3). Importantly, the refolding assay was not appreciably inhibited by Scythe protein lacking the BAG domain (Scythe ΔC) (Figure 3). These data strongly suggest that Scythe has the previously unanticipated ability to act in a BAG-1-like manner to inhibit Hsp70-mediated protein refolding.

Reaper relieves Scythe-mediated inhibition of Hsp70

Although BAG-1 protein clearly inhibits Hsp70's protein folding activity, its mode of regulation has yet to be elucidated. It has been postulated that BAG-1-mediated repression of Hsp70 must be reversible by as yet undiscovered ligands. As Scythe was originally identified as a Reaper ligand, we wished to determine whether Reaper might reverse Scythe's antagonistic effect on Hsp70-mediated protein folding. To test this, we added increasing amounts of Reaper to the *in vitro* β-galactosidase refolding assay. As shown in Figure 4A, Reaper was able to relieve Scythe's inhibition of Hsp70 refolding, with 80% reversal at a 1:4 (Scythe:Reaper) molar ratio. It is unclear whether this molar ratio reflects true complex stoichiometries because a significant fraction

of bacterially produced Reaper protein may be insoluble/non-functional. Nonetheless, similar amounts of Reaper had no effect on BAG-1-mediated inhibition of Hsp70, indicating that the reversal was specific for Scythe (Figure 4B). These data demonstrate that Reaper binding provides an effective means of reversing Scythe's inhibition of Hsp70 function.

Reaper inhibits the physical association of Scythe and Hsp70

Since a Scythe mutant unable to physically bind Hsp70 (ΔC Scythe) could not inhibit Hsp70-mediated protein refolding, we postulated that Reaper might act by rendering Scythe unable to bind Hsp70. To examine this issue, we conducted a series of protein binding studies using recombinant Reaper, Scythe and Hsp70 proteins. His-tagged Scythe was incubated with Hsp70 in the presence of increasing amounts of recombinant Reaper. As shown in Figure 5A, Reaper effectively inhibited the Scythe–Hsp70 interaction, while having a considerably less substantial effect on the BAG-1–Hsp70 association. These results are consistent with Reaper's ability to reverse the functional effects of Scythe, but not BAG-1, on Hsp70-mediated protein re-folding. A similar reversal of Scythe–Hsc70 binding was observed when we added recombinant GST–Reaper protein, but not GST alone, to Scythe immunoprecipitates from 293 cells (Figure 5B). Collectively, these data demonstrate that Reaper binding to Scythe both displaces Hsc70 and reverses Scythe-mediated inhibition of Hsp70 function.

The BAG domain is required for sequestration of Scythe-associated cytochrome c-releasing activity

As described above, addition of recombinant Reaper to cell-free extracts of *Xenopus* eggs triggers a number of biochemical hallmarks of apoptosis, including mitochondrial cytochrome *c* release, caspase activation, and internucleosomal DNA cleavage and fragmentation of added nuclei. As we reported previously, addition of excess recombinant Scythe to *Xenopus* egg extracts inhibits Reaper-induced apoptosis. We therefore speculated that the exogenously added Scythe protein could re-sequester cytochrome *c*-releasing factors dissociated from endogenous Scythe by Reaper. In agreement with this interpretation, the material released from Scythe immunoprecipitates by Reaper could no longer induce cytochrome *c* release if first incubated with recombinant Scythe protein (Thress *et al.*, 1999). In order to determine whether the ability of Scythe to interact with Hsc70 is important for its ability to sequester cytochrome *c*-releasing factor(s), egg extracts were supplemented with Reaper in combination with either wild-type human Scythe or Scythe unable to bind Hsc70 (ΔC Scythe). Under these conditions, the wild-type protein, but not the ΔC mutant Scythe, markedly dampened caspase activation and mitochondrial cytochrome *c* release in response to Reaper addition (Figure 6A and B). Importantly, while ΔC Scythe was unable to bind Hsc70 and inhibit Reaper-induced caspase activation, the ΔC and wild-type Scythe proteins bound Reaper to a similar extent (Figure 6C). Furthermore, the BAG domain of Scythe seems to be sufficient for these effects, as GST protein fused to the isolated BAG domain from Scythe (Scythe BAG) was as
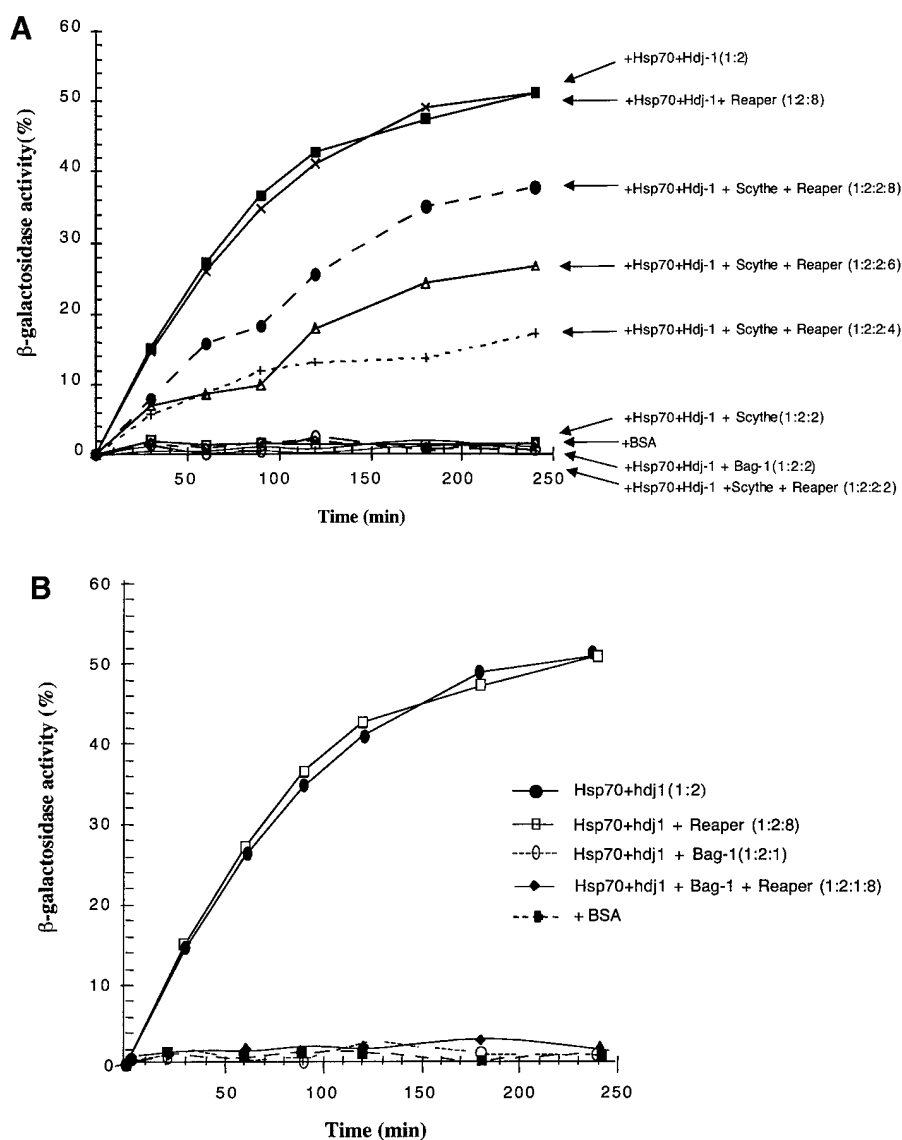
A

β-galactosidase activity(%) vs Time (min)

+Hsp70+Hdj-1(1:2)
+Hsp70+Hdj-1+ Reaper (12:8)
+Hsp70+Hdj-1 + Scythe + Reaper (1:2:2:8)
+Hsp70+Hdj-1 + Scythe + Reaper (1:2:2:6)
+Hsp70+Hdj-1 + Scythe + Reaper (1:2:2:4)
+Hsp70+Hdj-1 + Scythe(1:2:2)
+BSA
+Hsp70+Hdj-1 + Bag-1(1:2:2)
+Hsp70+Hdj-1 +Scythe + Reaper (1:2:2:2)

B

β-galactosidase activity (%) vs Time (min)

● Hsp70+hdj1(1:2)
▢ Hsp70+hdj1 + Reaper (1:2:8)
○ Hsp70+hdj1 + Bag-1(1:2:1)
◆ Hsp70+hdj1 + Bag-1 + Reaper (1:2:1:8)
▪ + BSA

Fig. 4. Reaper specifically relieves Scythe-mediated inhibition of Hsp70. (A) The experiment shown in Figure 3 was repeated with 3.2 μM human Scythe in the presence of increasing concentrations of Reaper (3.2–12.8 μM) to examine the reversal of Scythe-mediated inhibition on Hsp70-mediated refolding. (B) The inhibitory effect of BAG-1 (1.6 μM) on refolding was observed in the presence or absence of 12.8 μM Reaper, demonstrating that Reaper-induced reversal is specific for Scythe. As a control for spontaneous refolding, denatured β-galactosidase (3.2 nM) was diluted into refolding buffer containing 3.2 μM BSA.

effective as excess full-length Scythe in abrogating Reaper-induced caspase activation and cytochrome *c* release (Figure 6A and B). These data suggest that the BAG domain, required for both Hsc70 binding and inhibition of Hsc70/Hsp70-mediated protein folding is also required for effective re-sequestration of the apoptosis-inducing factors released from endogenous Scythe by Reaper.

Discussion

Scythe is a Reaper-interacting protein critical for Reaper-induced apoptosis in *Xenopus* egg extracts. In this report, we demonstrate that Scythe is also a modulator of Hsc70/Hsp70, able to inhibit chaperone-mediated protein folding. Reaper, in turn, inhibits this activity of Scythe. These

findings raise the intriguing possibility that regulation of protein folding plays an important role in control of apoptosis by Reaper.

Reaper reversal of Scythe-mediated Hsp70 repression

Although BAG-1 was previously shown to inhibit Hsp70 function, a ligand able to associate with the BAG-1–Hsp70–substrate complex, dissociate BAG-1 and allow resumption of protein folding was only speculated (Bimston *et al.*, 1998). Clearly, if BAG-1, or molecules like it, are to be considered viable regulators of protein homeostasis, the inhibition of Hsp70 must be reversible. In this report, we have identified Reaper as a ligand capable of reversing Scythe-mediated inhibition of Hsp70. Coincident with this reversal, Scythe is displaced from
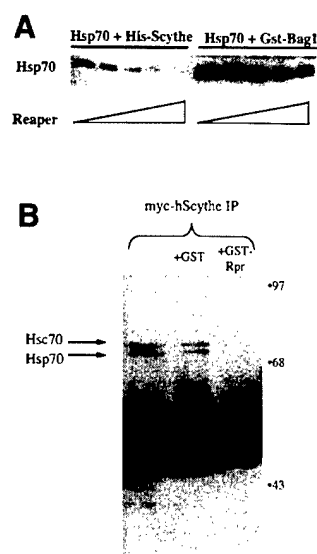
Fig. 5. Reaper specifically inhibits the physical association of Scythe and Hsp70. (**A**) His-Scythe (1 µM) or GST–BAG-1 (1 µM) was incubated with Hsp70 (1 µM) in refolding buffer. After complex formation, increasing concentrations (0, 2, 4, 8, 10 µM) of Reaper were added. Bound proteins were precipitated with either Ni⁺-agarose (His-Scythe) or glutathione–Sepharose (GST–BAG-1), washed, resolved by SDS–PAGE and processed for western blotting using Hsp70 monoclonal antibody 5a5. (**B**) 293T cells were transfected with 5 µg of myc-tagged human Scythe (myc-hScythe). Thirty-six hours after transfection, cells were lysed and centrifuged, and supernatants were incubated with recombinant GST or GST–Reaper (GST–Rpr) for 30 min at 4°C. Subsequently, the lysates were incubated with a monoclonal myc antibody for 1 h at 4°C. PAS beads were then added and, after an additional 1 h incubation, the beads were pelleted, washed three times in lysis buffer, and bound proteins were resolved by SDS–PAGE. After western transfer, the blots were probed with an anti-Hsc70 monoclonal antibody.

Hsp70, in accordance with the hypothetical scheme originally proposed for BAG-1. Interestingly, Reaper binds to Scythe at a site distinct from the BAG domain (between amino acids 235 and 312 of Scythe); therefore, the displacement of Hsc70 from Scythe is not the result of competitive binding of Reaper to the same site (C.Holley, K.Thress and S.Kornbluth, unpublished observations). Rather, we hypothesize that Reaper promotes a conformational change in Scythe, leading to dissociation of Hsp70.

Scythe–Hsp70 and apoptotic regulation

How does Scythe-mediated inhibition of Hsp70 protein folding relate to the ability of Scythe/Reaper to regulate apoptosis? Building on the model originally proposed for BAG-1 (Figure 7), we hypothesize that the cytochrome c-releasing factor(s) sequestered by Scythe (denoted as 'X' in Figure 7) is held in a soluble, partially folded state through binding to Hsc70–Scythe complexes. Although we do not yet know whether cytochrome c-releasing factors form direct contacts with both Scythe and Hsp70, Scythe, like BAG-1, binds to the ATPase domain of Hsp70 (Figure 2F), making the substrate-binding domain of Hsp70 potentially available for binding 'X' or similar molecules. Moreover, if Scythe functions mechanistically like BAG-1, it is likely that the contact between Hsc70/Hsp70 and 'X' is direct. Upon binding to Reaper, Scythe–Hsp70 complexes dissociate; according to our

model, Hsp70 thus relieved of its inhibition then goes on to fold 'X', leading to cytochrome c release, caspase activation, etc.

Superficially, Hsc70 seems to fit the description of 'X' itself, i.e. a protein bound to Scythe and dissociated by addition of Reaper. However, multiple experiments have failed to reveal any direct cytochrome c-releasing activity of recombinant Hsc70 protein (data not shown). Given the abundance of Hsp70 family members in the cell, this is not surprising. Nonetheless, the ability of Scythe to interact with Hsc70 appears to be important for its ability to sequester cytochrome c-releasing factor(s); a Scythe molecule able to interact with Reaper (data not shown), but lacking the BAG domain, could not behave like excess wild-type Scythe in preventing Reaper-induced apoptosis.

Although we have not yet definitively identified the Scythe-associated cytochrome c-releasing factors, the only factors thus far demonstrated to have direct cytochrome c-releasing activity are pro-apoptotic members of the bcl-2 family (Desagher *et al.*, 1999; Gross *et al.*, 1999; Shimizu *et al.*, 1999). That 'X' may indeed be a bcl-2 family member is supported by several observations. First, the cytochrome c-releasing activity of 'X' released from Scythe can be abrogated by incubation with recombinant bcl-xL, an anti-apoptotic bcl-2 family member that can act through heterodimerization with its pro-apoptotic counterparts (Zha *et al.*, 1997). Secondly, we have recently identified a relatively well conserved BH3 domain in the C-terminus of Scythe (M.Olson and S.Kornbluth, unpublished observations). BH domains (bcl-2-like hetero-dimerization domains), of which there are four types $(BH_1–BH_4)$, are contiguous sequences shown to be critical for the association of bcl-2 family members (reviewed in Gross *et al.*, 1999). Although BH3-containing proteins may, themselves, be cytochrome c-releasing factors, Scythe alone does not seem to have such an activity. We speculate, therefore, that the BH3 domain of Scythe is a docking site for a heterodimerized pro-apoptotic bcl-2 family member. This possibility is currently under investigation. However, since bcl-2 family members may form higher order multimers (e.g. for formation of pores in mitochondrial membranes), the role of Scythe-bound Hsc70 may, in this context, be to assemble higher order complexes, rather than to properly fold monomeric 'X' (Adams and Cory, 1998; Lewis *et al.*, 1998).

Although our studies have thus far been confined to analysis of a *Drosophila* protein (Reaper) in a vertebrate system (*Xenopus* egg extracts), we note that the function of Scythe and its BAG domain may ultimately prove to be important in the context of fly apoptosis. As has recently been described, the *Drosophila* genome contains an apparent Scythe homolog (Adams *et al.*, 2000; Jasny, 2000). Moreover, in preliminary studies we have found that *in vitro* translated fly Scythe can bind to fly Reaper (K.Thress and S.Kornbluth, unpublished).

Regulation of cellular signaling through modulation of Hsp70/Hsc70

Although Hsp70 family chaperones are abundant in the cell, proteins like BAG-1 and Scythe may confer substrate specificity on these proteins, promoting protein folding/assembly of bound substrates in a regulated manner. Held in an inactive or sequestered state, Hsc70 substrates bound
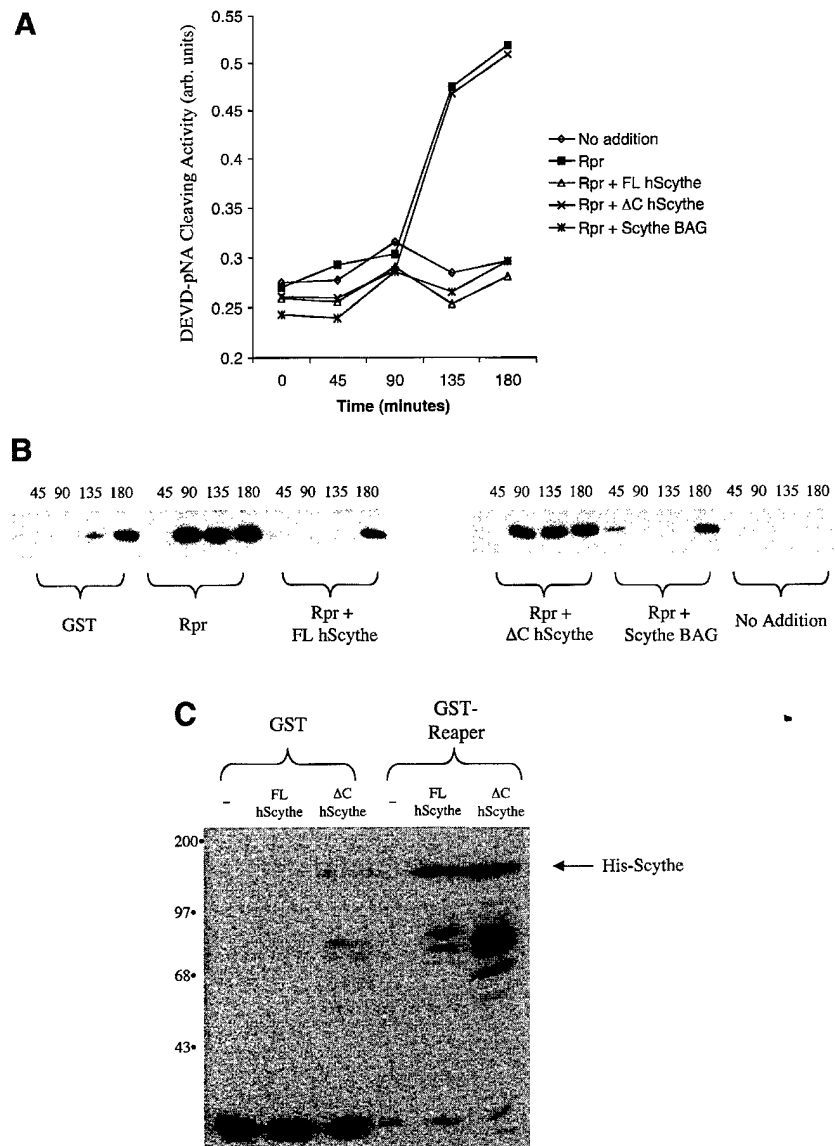
Fig. 6. BAG-deficient human Scythe is unable to protect against Reaper-induced apoptosis. (A) Recombinant Reaper (Rpr) protein (300 ng/μl) was added to *Xenopus* egg extracts in combination with equivalent amounts of recombinant full-length human Scythe (FL hScythe), BAG-deficient human Scythe (ΔC hScythe) or just the BAG domain of human Scythe (Scythe BAG). At the indicated times, 2 μl aliquots of extract were analyzed for caspase activity by cleavage of the artificial caspase substrate, DEVD-pNA. Following cleavage, released pNA was measured spectrophotometrically. (B) Samples were processed as in (A), but 15 μl aliquots were filtered through a 0.1 μM microfilter to remove particulate components, including mitochondria, and samples were processed for immunoblotting with an anti-cytochrome *c* monoclonal antibody. (C) Recombinant GST or GST–Reaper fusion proteins immobilized on glutathione–Sepharose beads were added to *Xenopus* egg extract and incubated at 4°C in the presence of equivalent amounts of either His-tagged full-length human Scythe (FL hScythe) or BAG-deficient Scythe (ΔC hScythe) proteins. After 1 h, the beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer, and processed for immunoblotting using a monoclonal penta-His antibody.

to Scythe/BAG-like proteins could be tightly controlled by binding of specific ligands able to dissociate Scythe–Hsc70 or BAG-1–Hsc70 complexes. That these reactions would have the requisite specificity is highlighted by the fact that Reaper was able to reverse Scythe-mediated inhibition of Hsp70 protein folding, while having no effect on similar inhibition by BAG-1. As alluded to earlier, BAG-1 binds a number of cellular signaling molecules; it is not known whether Scythe is similarly diverse in its binding partners. Moreover, it is not clear whether distinct ligands might have differential effects on different BAG–substrate or Scythe–substrate complexes. Nonetheless, regulatory networks of ligand–

co-chaperone–Hsc70 proteins as exemplified by Reaper–Scythe–Hsc70 offer a novel means to regulate the activity of cell signaling molecules critical for cell proliferation, cell death or cellular responses to stress.

Materials and methods

Preparation of Xenopus egg extracts

For induction of egg laying, mature female frogs were injected with 100 U of Pregnant Mare Serum Gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (HCG; USB). Fourteen to twenty hours after injection with HCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine pH 7.8,
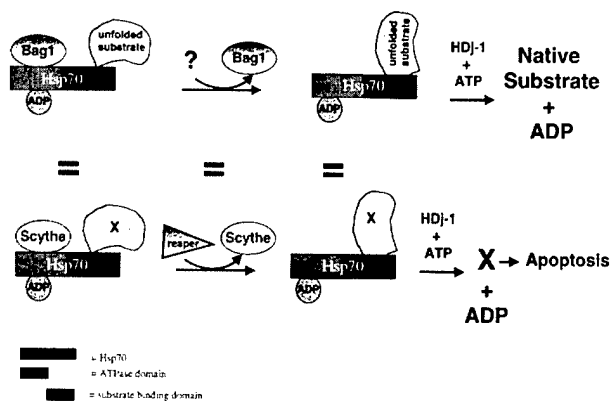
Fig. 7. Model for Reaper/Scythe function. BAG-1 binds to the ATPase domain of Hsp70 and inhibits its ability to mediate protein folding. In the presence of a hypothetical ligand, BAG-1 is released from Hsp70, promoting release of native substrate. In the case of Scythe we hypothesize that a similar series of events occurs; however, Reaper serves as the ligand to trigger Scythe dissociation from the Hsp70 complex. According to this speculative model, 'X' is released in its native form and can then trigger mitochondrial cytochrome *c* release and caspase activation. The figure has been adapted from Bimston *et al.* (1998).

washed three times in modified Ringers solution (1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 25 mM CaCl₂, 5 mM HEPES pH 7.8, 0.8 mM EDTA), and then washed in ELB [250 mM sucrose, 2.5 mM MgCl₂, 1.0 mM dithiothreitol (DTT), 50 mM KCl, 10 mM HEPES pH 7.4]. Eggs were packed by low speed centrifugation at 400 *g*. Following addition of aprotinin and leupeptin (final concentration 5 μg/ml), cytochalasin B (final concentration 5 μg/ml) and cycloheximide (final concentration 50 μg/ml), eggs were lysed by centrifugation at 10 000 *g* for 15 min. For nuclear formation, extracts were supplemented with demembranated sperm chromatin (1000 nuclei/μl) and an ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP and 50 mg/ml creatine phosphokinase). Recombinant proteins added to extracts were diluted in XB buffer (50 mM sucrose, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM K–HEPES pH 7.7) and added at a concentration of 300 ng/μl, unless otherwise indicated.

Preparation of GST fusion proteins

Full-length *Xenopus* Hsc70 was PCR amplified using the following primers: 5′-GATCTCTAGACATGTCTAAGGGACCAGCAGTT-3′ and 5′-GATCCTCGAGTTAGTCAACCTCCTCAATAGT-3′. PCR fragments were cloned into the *Xba*I–*Xho*I sites of the expression vector Gex KG, a derivative of Gex 2T (Pharmacia) containing additional polylinker sites and a polyglycine insert, and transformed into the Topp 1 bacterial strain (Stratagene). Recombinant protein was produced as previously described (Evans *et al.*, 1997). Full-length *Drosophila* Reaper and the C-terminal 312 amino acids of Scythe (Scythe C312) fused with GST were produced in a similar manner and constructed as described in Thress *et al.* (1998). GST fusions of human BAG-1 and Hsp70 were produced as previously described (Bimston *et al.*, 1998).

Baculovirus production of human Scythe protein

Full-length human Scythe (FL hScythe) and a truncated hScythe lacking the C-terminal 81 amino acids (hScythe ΔC) were both PCR amplified to possess a C-terminal His₆ tag and cloned into the *Xba*I–*Xho*I sites of the pFastBac vector. Protein was produced using the Bac-to-Bac Baculovirus Expression System (Gibco). Briefly, the resulting hScythe-pFastBac donor plasmids were transformed into DH10Bac *Escherichia coli* cells. *Escherichia coli* containing recombinant bacmid were cultured and recombinant bacmid DNA was recovered using a standard miniprep protocol. SF-9 insect cells were transfected with the bacmid DNA using CellFECTIN reagent (Gibco), incubated for 48 h at 27°C, and resulting recombinant baculovirus particles were harvested. Subsequently, SF-9 cells (2 × 10⁶ cells/ml) were infected with baculovirus for 48 h, washed twice in phosphate-buffered saline and lysed by dounce homogenization in HBS [10 mM HEPES pH 7.5, 20 mM β-glycerolphosphate, 150 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl

fluoride (PMSF) and 10 μg/ml each of pepstatin, chymostatin and leupeptin]. Lysate was then centrifuged at 4°C for 10 min at 10 000 r.p.m., and the supernatant was incubated with 1 ml of Ni-NTA agarose (Qiagen) for 30 min at 4°C. The beads were washed in 50 vols of HBS and eluted with HBS containing 200 mM imidazole in five fractions of 500 μl each.

Cell culture and transfections

FL hScythe, hScythe lacking the N-terminal 87 amino acids (hScythe ΔN) or hScythe ΔC was PCR amplified and cloned into a modified pcDNA3.1 mammalian expression vector (Invitrogen) containing an in-frame C-terminal myc tag. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). Cells (5 × 10⁵) were plated onto 60 mm dishes and 24 h later transfected with 3 μg of the appropriate myc-tagged hScythe construct or vector alone DNA using the Fugene transfection reagent (Roche biochemicals) as per manufacturer's instructions.

Immunoprecipitations

Thirty-six hours after transfection, cells were harvested in lysis buffer (10 mM Tris pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2% Tween-20, 10% glycerol) and lysates were incubated with anti-myc monoclonal antibody (Santa Cruz) for 2 h at 4°C. PAS beads were added to lysates, incubated for an additional 1 h, pelleted and washed three times in lysis buffer. Bound proteins were solubilized with sample buffer and resolved by SDS–PAGE. Resolved proteins were transferred to PVDF, blotted with an anti-Hsp70/Hsc70 monoclonal antibody (Affinity Bioreagents), incubated with HRP-linked goat anti-mouse secondary antibody and detected using the ECL system (Amersham).

Protein refolding assays

Protein refolding assays were conducted as previously described (Freeman *et al.*, 1996).

DEVDase assay

To measure caspase activity, 3 μl of each sample were incubated with 90 μl of assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric substrate Ac-DEVD-pNA (final concentration 200 mM: Biomol Caspase-3 assay system) at 37°C. At various time points, absorbance was measured at 405 nm in a LabSystems MultiSkan MS microtiter plate reader.

Acknowledgements

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